Regulation of the microglial NADPH oxidase by neurotransmitters: implications for microglial – neuronal interactions

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I, Emma Louise Mead, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Neurotransmitter dysregulation and reactive oxygen species (ROS) are a hallmark of neurodegenerative disease. Microglia, the immune cells of the CNS, express three NADPH oxidase isoforms (Nox1, Nox2 and Nox4), which produce superoxide that is used as an intracellular signalling molecule, mediating the production of neurotoxic and neurotrophic factors. Microglia also express a range of neurotransmitter receptors, enabling them to respond to physiological and pathological levels of neurotransmitters. As both microglial superoxide production and neurotransmitter dysregulation are common to many neurodegenerative conditions, the interaction between microglial neurotransmitter receptor modulation and NADPH oxidase activation was investigated.

Superoxide production was assessed in primary and BV2 microglia using flow cytometry, HPLC, fluorescence microscopy, and a colorometric assay. Glutamate (1 μM), GABA (100 μM) or BzATP (250 μM) induced NADPH oxidase derived superoxide production. Furthermore, antagonism of the group I mGluRs induced Nox1 and Nox2 dependent superoxide production through PKC / PI3-K pathways and p44/42ERK activation. Activation of mGluR3 induced Nox2 and Nox4 activation in a p44/42ERK and p38MAPK dependent manner, whilst activation of the group III mGluRs induced Nox2 and Nox4 activation dependent on p38MAPK signalling. Microglial NMDA receptor activation promoted superoxide production that was dependent on p38MAPK activation. Modulation of the microglial glutamate receptors mediated protection of cerebellar granule neurons, as deomstrated using microglial conditioned media assays. Activation of the microglial $GABA_A$ receptor induced Nox1 activation through PKC and p38MAPK signalling which mediated TNFα release and neurotoxicity, whilst P2Y2/4 receptor activation mediated Nox1 activation through PI3-K and p38MAPK with neurotoxic consequences. The findings in this thesis show that microglial Nox2 and Nox4 activation has neuroprotective consequences, whereas

microglial Nox1 activation mediates neurotoxicity. Modulation of the microglial neurotransmitter receptors therefore has ramifications for the survival of neurons in degenerative diseases, which could have important consequences for the production of future therapies for neurodegenerative conditions.

Table of Contents

Title ……...1

Declaration ……2

Acknowledgements ……...3

Abstract……..4

List of Figures and Tables ……10

List of Abbreviations ……...16

1. Introduction……..21

1.1 Cells of the Central Nervous System 22

1.1.1 Neurons.....222

1.1.2 Glia 255

1.1.2.1 Astrocytes 26

1.1.2.2 Oligodendrocytes.....28

1.1.2.3 Ependymal cells.....30

1.1.2.4 Brain macrophages 30

1.2 Microglia in heath and disease 31

1.3 Micrioglial activation in neurodegeneration.....35

1.4 The immune system 37

1.4.1 The brain as an immune privileged organ 37

1.5 Reactive oxygen species in the central nervous system.....41

1.5.1 Pathology and physiology of ROS and the immune response.....42

1.5.2 Production of superoxide by the NADPH oxidase 44

1.5.3 NADPH oxidase isoforms 48

1.5.4 NADPH oxidase in the CNS 50

1.5.4.1 Microglial expression of the NADPH oxidase 51

1.5.4.1.1 Microglial NADPH oxidase in physiological conditions.....52

1.5.4.1.2 Microglial NADPH oxidase in pathology 54

1.6 Neurotransmitter and neurotransmitter receptor dysregulation in neurodegeneration .60

1.6.1 Glutamate.....61

1.6.2 GABA 63

1.6.3 ATP.....64

1.6.4 Neurotransmitter receptor modulation of microglial activation in neurodegeneration and involvement of ROS 67
1.7 Aims and objectives 80
2. Materials and Methods 82
2.1 Materials 83
2.2 Methods 85
2.2.1 Cell Culture 85
2.2.1.1 BV2 cell culture 85
2.2.1.2 Primary microglial culture 86
2.2.1.3 Cerebellar Granule Cell (CGC) culture 889
2.2.2 Cell treatments 90
2.2.2.1 Treatment of primary and BV2 microglia 90
2.2.2.2 Treatment of CGCs 92
2.2.2.3 Conditioned media assays 92
2.2.3 Cell imaging 93
2.2.3.1 Immunocytochemistry 94
2.2.3.2 Assessment of cell death 95
2.2.4 Measurement of superoxide production 96
2.2.4.1 NBT detection of intracellular superoxide 98
2.2.4.1.1 NBT assay optimisation 100
2.2.4.1.2 Optimised NBT assay protocol 105
2.2.4.2 Dihydroethidium detection of intracellular superoxide – assessment by fluorescence microscopy 105
2.2.4.2.1 Optimisation of dHEth detection of intracellular superoxide by fluorescence microscopy 107
2.2.4.2.2 Optimised dHEth detection of intracellular superoxide by fluorescence microscopy protocol 110
2.2.4.3 Flow cytometry analysis of superoxide production 111
2.2.4.3.1 Optimisation of flow cytometry 112
2.2.4.3.2 Optimised flow cytometry method 115
2.2.4.4 HPLC detection of superoxide and hydrogen peroxide 116
2.2.4.4.1 Preparation of standards 117
2.2.4.4.2 Preparation of cells 121
2.2.4.4.3 HPLC detection of 2-OH-E^+ and Eth 122

2.2.5 NADPH oxidase activity assay....125
2.2.5.1 Optimised protocol for NADPH oxidase activity assay....125
2.2.6 Expression analysis....127
2.2.6.1 mRNA analysis using reverse transcription and polymerase chain reaction....127
2.2.6.1.1 RNA extraction....128
2.2.6.1.2 Reverse transcription....129
2.2.6.1.3 Polymerase Chain Reaction (PCR)....130
2.2.6.1.4 Resolution of PCR products....134
2.2.6.2 Western blotting....134
2.2.6.2.1 Cell lysis and sample preparation....135
2.2.6.2.2 Bradford assay....136
2.2.6.2.3 SDS-PAGE....136
2.2.6.2.4 Transfer....137
2.2.6.2.5 Immunoblotting....138
2.2.7 Enzyme linked immunosorbent assays (ELISA) of TNFα release....140
2.2.8 Statistical analysis....141
3. Neurotransmitter induction of Superoxide productuion in microglia....142
3.1 Introduction and summary of results....143
3.2 Results....146
3.2.1 Glutamate, GABA and ATP induce superoxide production in microglia which is due to NADPH oxidase activation....146
3.2.2 Neurotransmitter receptor agonists and antagonists induce superoxide production in microglia as a consequence of NADPH oxidase activation....156
3.2.2.1 Modulation of mGluRs induces superoxide production in microglia....156
3.2.2.2 Modulation iGluRs induces superoxide production in microglia....167
3.2.2.3 Modulation of GABA receptors induces superoxide production in microglia 1744
3.2.2.4 Modulation of the metabotropic purinergic receptors induces superoxide production in microglia....185
3.3 Discussion....192
3.3.1 Neurotransmitters modulate superoxide production in microglia....192
3.3.2 Modulation of neurotransmitter receptors induces superoxide production in microglia....196
3.3.3 Conclusions....206

4. Neurotransmitter induction of NADPH oxidase isoforms and microglial signalling pathways implicted in NADPH oxidase isoform activity.....207

4.1 Introduction and summary of results.....208

4.2 Results.....211

4.2.1 Neurotransmitters induce expression and activity of Nox1, Nox2 and Nox4 .211

4.2.2 Neurotransmitter receptor modulation affects the expression and activity of Nox1, Nox2, and Nox4.....218

4.2.3 MAPK signalling in neurotransmitter receptor modulated NADPH oxidase activation 231

4.2.4 Microglial activation is not modulated by neurotransmitter induced superoxide production.....248

4.3 Discussion 252

4.3.1 Neurotransmitter and receptor induced NADPH oxidase isoform expression and activity in microglia 253

4.3.2 The MAPK signalling pathway is implicated in neurotransmitter receptor induced NADPH oxidase activity.....263

4.3.3 Neurotransmitter induced NADPH oxidase activity and microglial reactivity...270

4.3.4 Conclusions 271

5. Ramifications of neurotransmitter and receptor mediated microglial NADPH oxidase activity on neuronal survival.....272

5.1 Introduction and summary of results.....273

5.2 Results.....277

5.2.1 Microglia express and release TNFα in an NADPH oxidase dependent manner following treatment with neurotransmitters and receptor agonists or antagonists 277

5.2.2 Microglial conditioned media (MGCM) from microglia treated with neurotransmitters and receptor agonists or antagonists modulates neuronal survival in an NADPH oxidase dependent manner.....287

5.2.3 MGCM from microglia treated with neurotransmitters and receptor agonists or antagonists modulates neuronal survival after treatment with staurosporine, a model of neuronal apoptosis, in an NADPH oxidase dependent manner.....311

5.3 Discussion 324

5.3.1 Neurotransmitter mediated microglial TNFα release and ramifications for neuronal survival.....324

5.3.2 Receptor mediated microglial TNFα production and ramifications for neuronal survival 329

5.3.3 Conclusions 341

6. General Discussion 343
6.1 Modulation of microglial neurotransmitters induces NADPH oxidase activation with ramifications for neuronal survival 344
6.2 Conclusions 354
6.3 Suggested future work 356
References 358

List of Figures and Tables

Figure 1.1 Schematic of a neuron 23
Figure 1.2 Cells and circuitry of the cerebellum 25
Figure 1.3 The lactate shuttle 27
Figure 1.4 Schematic of an oligodendrocyte myelinating axons 29
Figure 1.5 Microglial activation states 34
Figure 1.6 The blood brain barrier 39
Figure 1.7 Superoxide production from NADPH oxidase activation 46
Figure 1.8 Assembly of the NADPH oxidase 47
Figure 1.9 The structure of Nox1, Nox2 and Nox4 50
Figure 1.10 Known ramifications of NADPH oxidase activation 53
Figure 1.11 Summary of activation of the NADPH oxidase following modulation of neurotransmitter receptors 78
Figure 2.1 Microglial preparation using a percoll gradient 87
Figure 2.2 Microglia stained with Isolectin B_4 and Hoescht 33342 88
Figure 2.3 Hoescht 33342 and Propidium Iodide staining of CGC's to assess cell death 96
Figure 2.4 Reduction of NBT to $NBTH_2$ formazan precipitate 99
Figure 2.5 Time course analysis of superoxide production by NBT reduction 101
Figure 2.6 Concentration titration of PMA and LPS 102
Figure 2.7 Concentration titration of apocynin 103
Figure 2.8 PMA, LPS or apocynin toxicity 104
Figure 2.9 Optimal conditions for the detection of superoxide production using the NBT assay 104
Figure 2.10 The oxidation of dihydroethidium to 2-OH-E^+ by superoxide 106
Figure 2.11 Time course analysis of dHEth fluorescence 108

Figure 2.12 Superoxide production in microglia treated with PMA or LPS in the presence and absence of apocynin can be detected by dHEth flourescence..... 109
Figure 2.13 Representative dot-plot from un-labelled BV2 microglia..... 113
Figure 2.14 FACS analysis of PMA induced superoxide production in BV2 microglia..... 114
Figure 2.15 HPLC chromatograms of 2-OH-E^+ (A), Ethidium (B) and dHEth (C)..... 119
Figure 2.16 Standard curves of 2-OH-E^+, Ethidium, and dHEth..... 120
Figure 2.17 Example chromatograms and HPLC analysis dHEth oxidation products from cells treated with PMA in the presence or absence of apocynin..... 124
Figure 2.18 Time course analysis of NADPH oxidase activity..... 127
Figure 2.19 The polymerase chain reaction..... 130
Figure 3.1 NBT analysis showing induction of superoxide production by neurotransmitters..... 148
Figure 3.2 Neurotransmitters induce microglial superoxide production – analysis by dHEth fluorescence imaging..... 150
Figure 3.3 Quantification of neurotransmitter induced superoxide production by flow cytometry measuring dHEth fluorescence and analysis of dHEth oxidation by HPLC..... 153
Figure 3.4 NADPH oxidase activity following treatment with neurotransmitters..... 155
Figure 3.5 NBT assay to investigate superoxide production following treatment of BV2 microglia with (A) group I, (B) group II and (C) group III mGluR agonists and antagonists..... 158
Figure 3.6 Dose-dependency NBT analysis of MTEP (A), NAAG (B) and L-AP4 (C) to determine the optimal concentration required to induce superoxide production in an NADPH oxidase dependent manner..... 160
Figure 3.7 dHEth fluorescence showing NADPH oxidase dependent superoxide production in primary microglia following treatment with MTEP, NAAG or L-AP4..... 162
Figure 3.8 Quantitative analysis of superoxide production following modulation of mGluRs by flow cytometry analysis of dHEth fluorescence and HPLC analysis of dHEth oxidation products..... 164
Figure 3.9 Modulation of mGluRs induces NADPH oxidase activity..... 166

Figure 3.10 Analysis of superoxide production in BV2 cells (Ai, ii) and primary microglia (Bi, ii and Ci, ii) following modulation of the NMDA and AMPA receptors..... 169

Figure 3.11 Quantative analysis of iGluR induced superoxide production by flow cytometry analysis of dHEth fluorescence and HPLC analysis of dHEth oxidation products..... 171

Figure 3.12 NADPH oxidase activity assay following modulation of the NMDA (A) and AMPA receptors (B)..... 173

Figure 3.13 Analysis of superoxide production in BV2 and primary microglia following modulation of the $GABA_A$ and $GABA_B$ receptors..... 176

Figure 3.14 Quantitative analysis of $GABA_A$ induced superoxide production by flow cytometry of dHEth fluorescence and HPLC analysis of dHEth oxidation products..... 178

Figure 3.15 NADPH oxidase activity after treatment of BV2 microglia with muscimol..... 180

Figure 3.16 Analysis of $GABA_A$ receptor subunit expression (A) and modulation of superoxide production following co-treatment of microglia with $GABA_A$ receptor agonists and antagonists (B, C)..... 183

Figure 3.17 Analysis of superoxide production in BV2 and primary microglia after modulation of the P2Y2/4 and P2Y1 receptors..... 187

Figure 3.18 Quantitative analysis of P2Y2/4 receptor induced dHEth fluorescence by flow cytometry and of dHEth oxidation products by HPLC..... 189

Figure 3.19 NADPH oxidase activity after treatment of BV2 microglia with UTPγS... 191

Figure 3.20 Possible mechanisms of NADPH oxidase activation following modulation of microglial glutamate receptors..... 201

Figure 3.21 Possible mechanisms by which modulation of the $GABA_A$ and P2Y2/4 receptors may induce superoxide production in microglia..... 205

Figure 4.1 NADPH oxidase isoform expression in primary microglia treated with the neurotransmitters glutamate, GABA or BzATP..... 212

Figure 4.2 Analysis of NADPH oxidase isoform activity after treatment of BV2 or primary microglia with glutamate (Ai, ii, iii), GABA (Bi, ii, iii) or BzATP (Ci, ii, iii)..... 215

Figure 4.3 Nox1, Nox2 and Nox4 expression in primary microglia after treatment

with neurotransmitter receptor agonists and antagonists..... 219

Figure 4.4 Modulation of NADPH oxidase isoform activity after treatment of microglia with the group I mGluR antagonist MTEP (Ai, ii, iii), the mGluR3 agonist NAAG (Bi, ii, iii) or the group III mGluR agonist L-AP4 (Ci, ii, iii)..... 223

Figure 4.5 Modulation of NADPH oxidase isoform activity after treatment of microglia with the $GABA_A$ receptor agonist muscimol (Ai, ii, iii) or the P2Y2/4 receptor agonist UTPγS (Bi, ii,iii)..... 226

Figure 4.6 Nox1, Nox2 and Nox4 expression in primary microglia after treatment with iGluR receptor agonists and antagonists..... 229

Figure 4.7 Inhibition of p44/42ERK or p38MAPK attenuates superoxide production and NADPH oxidase activity following modulation of microglial mGluRs..... 233

Figure 4.8 Inhibition of p44/42ERK or p38MAPK attenuates superoxide production and NADPH oxidase activity following modulation of the $GABA_A$ receptor or the P2Y2/4 receptor..... 236

Figure 4.9 Inhibition of p44/42ERK or p38MAPK attenuates superoxide production and NADPH oxidase activity following modulation of iGluRs..... 238

Figure 4.10 Neurotransmitter receptor induced superoxide production modulates p38MAPK and p44/42ERK phosphorylation..... 241

Figure 4.11 iGluR induced superoxide production modulates p38MAPK phosphorylation..... 244

Figure 4.12 iGluR induced superoxide production modulates p44/42ERK phosphorylation..... 247

Figure 4.13 Modulation of microglial reactivity after treatment with neurotransmitters..... 249

Figure 4.14 Microglial recactivity following modulation of neurotransmitter receptors..... 251

Figure 4.15 Schematic of mechanisms of neurotransmitter mediated Nox isoform activation..... 269

Figure 5.1 Schematic of known ramifications of microglial neurotransmitter receptor modulation..... 275

Figure 5.2 Production and release of TNFα by microglia treated with neurotransmitters..... 278
Figure 5.3 TNFα expression in primary microglia treated with neurotransmitter receptor agonists or antagonists..... 280
Figure 5.4 TNFα release from microglia treated with neurotransmitter receptor agonists or antagonists..... 283
Figure 5.5 TNFα expression in primary microglia after modulation of iGluRs..... 285
Figure 5.6 CGC death after treatment with MGCM from microglia treated with neurotransmitters in the presence or absence of apocynin..... 288
Figure 5.7 Analysis of CGC phospho-Akt expression after treatment with MGCM from microglia treated with neurotransmitters in the presence or absence of apocynin..... 293
Figure 5.8 CGC death after treatment with MGCM from microglia treated with mGluR agonists or antagonists in the presence or absence of apocynin.... 296
Figure 5.9 CGC death after treatment with MGCM from microglia treated with the $GABA_A$ receptor agonist in the presence or absence of apocynin..... 298
Figure 5.10 CGC death after treatment with MGCM from microglia treated with the P2Y2/4 receptor agonist in the presence or absence of apocynin..... 300
Figure 5.11 Analysis of CGC phospho-Akt expression after treatment with MGCM from microglia treated with neurotransmitter receptor agonists or antagonists in the presence or absence of apocynin..... 302
Figure 5.12 Analysis of caspase 12 cleavage in CGCs after treatment with MGCM from microglia treated with neurotransmitter receptor agonists or antagonists in the presence or absence of apocynin..... 304
Figure 5.13 Analysis of TNFR1 expression in CGCs after treatment with MGCM from microglia treated with neurotransmitter receptor agonists or antagonists in the presence or absence of apocynin..... 307
Figure 5.14 Modulation of microglial iGluRs affects neuronal survival..... 309
Figure 5.15 Neuronal apoptosis after treatment of CGCs Sts and MGCM from microglia treated with neurotransmitters in the presence or absence of apocynin..... 312
Figure 5.16 Neuronal apoptosis after treatment of CGCs Sts and MGCM from microglia treated with mGluR receptor agonists or antagonists in the

presence or absence of apocynin..... 316
Figure 5.17 Neuronal apoptosis after treatment of CGCs with Sts and MGCM from microglia treated with the $GABA_A$ receptor agonist in the presence or absence of apocynin..... 320
Figure 5.18 Neuronal apoptosis after treatment of CGCs with Sts and MGCM from microglia treated with the P2Y2/4 receptor agonist in the presence or absence of apocynin..... 322
Figure 5.19 Schematic of microglial mediated neuronal death or survival following exposure of microglia to neurotransmitters..... 329
Figure 5.20 Summary of the mechanisms of neuronal survival and death after modulation of the microglial NADPH oxidase following treatment with receptor agonists or antagonists..... 340
Figure 6.1 Microglial glutamate receptor induced superoxide productions and ramifications for neuronal survival..... 350
Figure 6.2 Activation of the microglial $GABA_A$ receptor or P2Y2/4 receptors are neurotoxic through activation of Nox1..... 353

Table 1. Neurotransmitter receptor expression on microglia..... 77
Table 2. Compounds used for treatment of BV2 and primary microglia..... 91
Table 3. Antibodies used for immunostaining 95
Table 4. Elution conditions for the analysis of dHEth and 2-OH-E^+ and ethidium oxidation products by HPLC with fluorescence detection..... 122
Table 5. PCR primers..... 133
Table 6. Composition of resolver gels for 10% and 12% gels and stacker gel..... 137
Table 7. List of primary and secondary antibodies used for Western blotting..... 139
Table 8. Summary table of NADPH oxidase activation and signalling cascades elicited by modulation of microglial neurotransmitter receptors..... 354

List of Abbreviations

·OH – Hydroxyl Radical

2-AG - 2-arachidonylglycerol

2-OH-E$^+$ - 2- Hydroxyethidium

ACPD - *trans*-(1*S*,3*R*)-1-Amino-1,3-dicarboxycyclopentane

AD – Alzheimer's Disease

ADP – Adenosine Diphosphate

AIDA - (*RS*)-1-Aminoindan-1,5-dicarboxylic acid

AIDS – Acquired Immune Deficiency Syndrome

ALS - Amylotrophic Lateral Sclerosis

AMPA - 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid

AngII – Angiotensin II

ANOVA – Analysis of Variance

APICA - (*RS*)-1-Amino-5-phosphonoindan-1-carboxylic acid

APS – Ammonium Persulphate

Ara-C - Cytosine Furanoarabinoside

ATF-1 - Activating Transcription Factor 1

ATP – Adenosine Tri-Phosphate

Aβ – Beta amyloid

BBB – Blood Brain Barrier

BDNF – Brain Derived Neurotropic Factor

BSA – Bovine Serum Albumin

CDPPB - 3-Cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide

CgA – Chromogranin A

CGC – Cerebellar granule cells

CHPG – **(***RS*)-2-Chloro-5-hydroxyphenylglycine

CNQX - 6-cyano-7-nitroquinoxaline-2,3-dione

CNS – Central Nervous System

CRB2 - cannabinoid receptor type 2

CSF – Cerebro-spinal Fluid

DA – Dopaminergic

DAG – Diacylglycerol

DAPI - 4'-6-Diamidino-2-phenylindole

DCG-IV - (2*S*,2'*R*,3'*R*)-2-(2',3'-Dicarboxycyclopropyl)glycine

DEPC – Diethylpyrocarbonate

dHEth – Dihydroethidium

DHPG - (*RS*)-3,5-Dihydroxyphenylglycine

DMEM - Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl Sulphoxide

DPI – Diphenylene Iodonium

DTPA - Diethylene Triamine Pentaacetic Acid

EBSS – Earle's Balanced Salt Solution

ECL – Enhanced Chemiluminescence

EDTA - Ethylene Diamine Tetraacetic Acid

EGTA - Ethylene Glycol Tetraacetic Acid

ELISA – Enzyme Linked Immunosorbant Assay

EMCV - Encephalomyocarditis Virus

ER – Endoplasmic Reticulum

ERK1/2 – Extracellular Signal Regulated Kinase 1/2

Eth – Ethidium

FACS – Fluorescence Activated Cell Sorting

FAD – Flavin Adenine Dinucleotide

FBS – Foetal Bovine Serum

FGF – Fibroblast Growth Factor

FITC - Fluorescein Isothiocyanate

FSC – Forward Scatter

GABA - Gamma-aminobutyric acid

GAD – Glutamic Acid Decarboxylase

GAS – Interferon Actviated Site

GDNF – Glial Derived Neurotrophic Factor

GFAP – Glial Fibrillary Associated Protien

GLAST- Glutamate Aspartate Transporter

GLT1 – Glutamate Transporter 1

GPCR – G Protein Coupled Receptor

GSH – Glutathione

H_2O_2 – Hydrogen peroxide

HD – Hunington's Disease

HGF – Hepatocyte Growth Factor

Hoescht - 2´-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5´-bi-1H-benzimidazole

HPLC – High Performance Liquid Chromatography

HRP – Horseradish Peroxidase

IFNγ – Interferon Gamma

iGluR – Ionotropic Glutamate Receptor

IL-1β – Interleukin-1β

IL-6 – Interleukin-6

iNOS – Inducible Nitric Oxide Synthase

IP_3 - Inositol (1,3,5) triphosphate

ISRE – Interferon Stimulated Response Element

JAK/STAT – Janus Kinase / Signal Transducer and Activator of Transcription

JNK – Janus Kinase

LAMP-1 - Lysosomal Associated Membrane Protein-1

L-AP4 - L-(+)-2-Amino-4-phosphonobutyric acid

LPS – Lipopolysaccharide

LTP – Long Term Potentiation

MAP4 - (*S*)-2-Amino-2-methyl-4-phosphonobutanoic acid

MAPK – Mitogen Activated Protien Kinase

MCPG - (*RS*)-α-Methyl-4-carboxyphenylglycine

MEM – Minimum Essential Medium

MGCM – Microglial Conditioned Media

mGluR – Metabotropic Glutamate Receptor

MHC II - Major Histocompatibilty Complex II

MIP1-α – Macrophage Inflammatory Protein 1-α

MK-801 - (5*R*,10*S*)-(-)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cylcohepten-5,10-imine-maleate

M-MLV – Murine Moloney Leukaemia Virus

MMP9 – Matrix Metalloproteinase 9

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRF-1 – Microglial Response Factor 1

MS – Multiple Sclerosis

MTEP - 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride

NAAG - *N*-Acetyl-L-aspartyl-L-glutamic acid

NADP – Reduced Nicotine Adenine Dinucleotide Phosphate

NADPH Oxidase – Nicotine Adenine Dinucleotide Phosphate Oxidase

NBT – Nitro Blue Tetrazolium chloride

NF-κB – Nuclear Factor Kappa-B

NGF – Nerve Growth Factor

NGS – Normal Goat Serum

NMDA - *N*-Methyl-D-aspartic acid

NO – Nitric Oxide

NSAI – Non-Steroidal Anti-Inflammatory

NT-3 – Neurotrophin-3

$O_2^{\cdot -}$ - Supreroxide

OAT – Optimal Annealing Temperature

OPC – Oligodendrocyte Precursor Cell

PACAP - Pituitary Adenylate Cyclase Activating Polypeptide

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PD – Parkinson's Disease

PDGF – Platelet Derived Growth Factor

PDL – Poly-D-Lysine

PFA – Paraformaldehyde

PI – Propidium Iodide

PI3-K – Phosphatidyl Inositol-3 Kinase

PIP_2 – Phosphatidylinositol 4,5-bisphosphate

PKC – Protien Kinase C

PLC – Phospholipase C

PMA – Phorbol 12-myristate 13- acetate

PMN – Polymorphonucelar Neutrophils

Ptx – Picrotoxin

PVDF – Polyvinylidine Fluoride

QA – Quisqualic Acid

RAGE – Receptor for Advanced Glycosylated End-products

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SBTI – Soybean Trypsin Inhibitor

SDS – Sodium Dodecyl Sulphate

SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SH3 – Src Homology 3 domain

SIP – Sample Injection Port

SKF-97541 – 3-Aminopropyl(methyl)phosphinic acid

SN – Substantia Nigra

SOD – Superoxide Dismutase

SR – Scavenger Receptor

SSC – Side Scatter

SVZ – Sub Ventricular Zone

TBE – Tris/Borate/EDTA Buffer

TBI – Traumatic Brain Injury

TGF-β – Transforming Growth Factor-β

TLR – Toll Like Receptor

TMED - N, N, N', N'-tetramethylethylenediamine

TNFR1 - TNF receptor 1

TNFR1 – Tumor Necrosis Factor Receptor 1

TNFR2 – Tumor Necrosis Factor Receptor 2

TNFα – Tumor Necrosis Factor α

TRITC - Tetramethyl Rhodamine Iso-thiocyanate

TRX – Thioredoxin

TTBS – Tween – Tris Buffered Saline

UDP – Uridine Diphosphate

UTP – Uridine Triphosphate

VRAC – Voltage Regulated Anion Channels

VSMC's – Vascular Smooth Muscle Cells

VZ – Ventricular Zone

Chapter 1

Introduction

1.1 Cells of the Central Nervous System

The central nervous system (CNS) is comprised of the brain and spinal cord, and is responsible for information processing and interactions with the external environment. The CNS is composed of a network of equal numbers of neuronal and non-neuronal cells, with an estimated 86.1 billion neurons and 84.6 billion non-neuronal cells (Azevedo et al. 2009), which interact in a bi-directional manner allowing the efficient transfer of action potentials throughout the CNS.

1.1.1 Neurons

Neurons receive, store, process and transfer information in the CNS. They are electrochemically excitable cells that communicate through the release and up-take of excitatory or inhibitory neurotransmitters, which elicit either excitatory action potentials or inhibitory signals depending on the receptors to which they bind (Seal & Edwards 2006). Neurons are composed of a soma (cell body), a number of dendrites (processes extending from the soma) where input to the neurons occurs, and a myelinated axon, which propagates the action potential (Fig. 1.1).

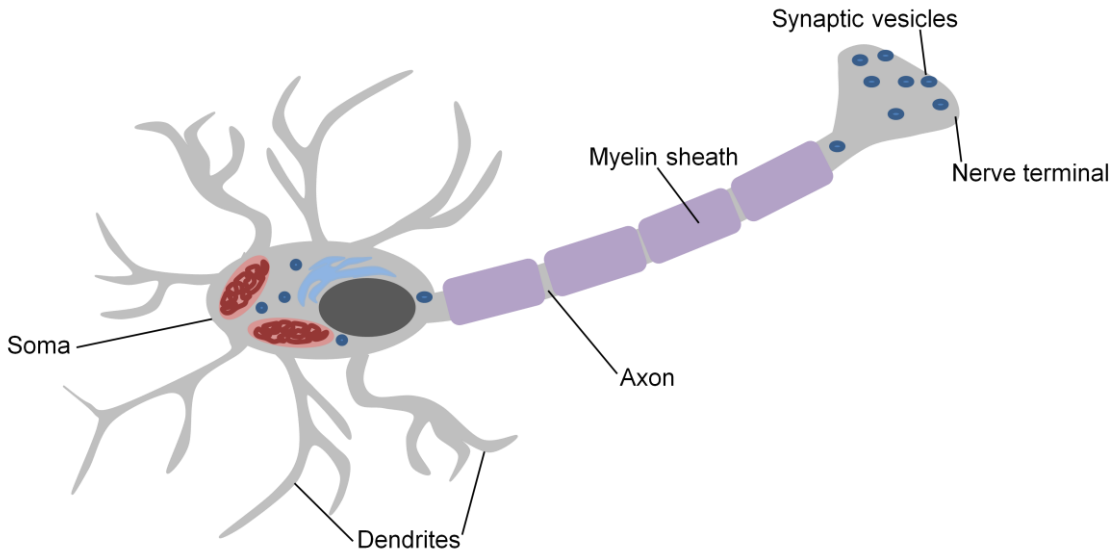

Figure 1.1 Schematic of a Neuron. *Neurons have a soma, multiple and highly branched dendrites, a single large axon, which is myelinated to aid propagation of the action potential, and a nerve terminal, which is rich in synaptic vesicles containing neurotransmitters, neuropeptides and other factors for release into the synaptic cleft.*

The action potential is induced by the depolarisation of the neuronal membrane, achieved by electrical input from adjacent cells through the dendrites. Axonal depolarisation induces an electrical signal that promotes neurotransmitter release from the synapse at the nerve terminal into the synaptic cleft, where post-synaptic neurons or effector cells are modulated (Catterall 1984).

The interactions between neurons comprise a complex network of neuronal pathways, which are crucial to the functioning of the CNS. Damage to neurons, or inhibition or breaks in neuronal networks underlies diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Multiple Sclerosis (MS) and stroke, along with several other neurological disorders, resulting in the manifestation of clinical symptoms.

Neuronal activity can be modelled *in vitro* using cell and slice cultures. In this thesis, cerebellar granule neurons have been used as a model of neuronal activity. The neurons of the cerebellum develop post-natally, therefore allowing the preparation of neurons from early post-natal rats. Cerebellar granule cells (CGCs) are predominantly glutamatergic (Pocock & Nicholls 1998), making these cells a useful model to study the neurotoxic effects of glia and neurotransmitter dysregulation, which underlies the pathogenesis of several neurodegenerative conditions, on neuronal survival.

The cerebellum co-ordinates voluntary movement and balance, and is crucial for the transfer of information to other parts of the brain. The cerebellum is composed of three distinct layers (Fig. 1.2). The molecular cell layer is comprised of basket cell interneurons and branching Purkinje cell dendrites, which enable synaptic activity. The Purkinje cell layer is composed of a single row of cell bodies, and finally, the granule cell layer consists of granule and Golgi interneurons. The main electrical input to the cerebellum comes from the mossy fibers, which form excitatory glutamatergic synapses with granule cells in the granule layer. Furthermore, the cerebellum also receives excitatory input from the climbing fibers of the brain stem, which form excitatory glutamatergic synapses with up to ten Purkinje cells. The Purkinje cells can process the excitatory inputs from the climbing and mossy fibers, and the inhibitory signals from the basket cells. In this way, Purkinje cells can relay neuronal signals to the cerebellar nuclei.

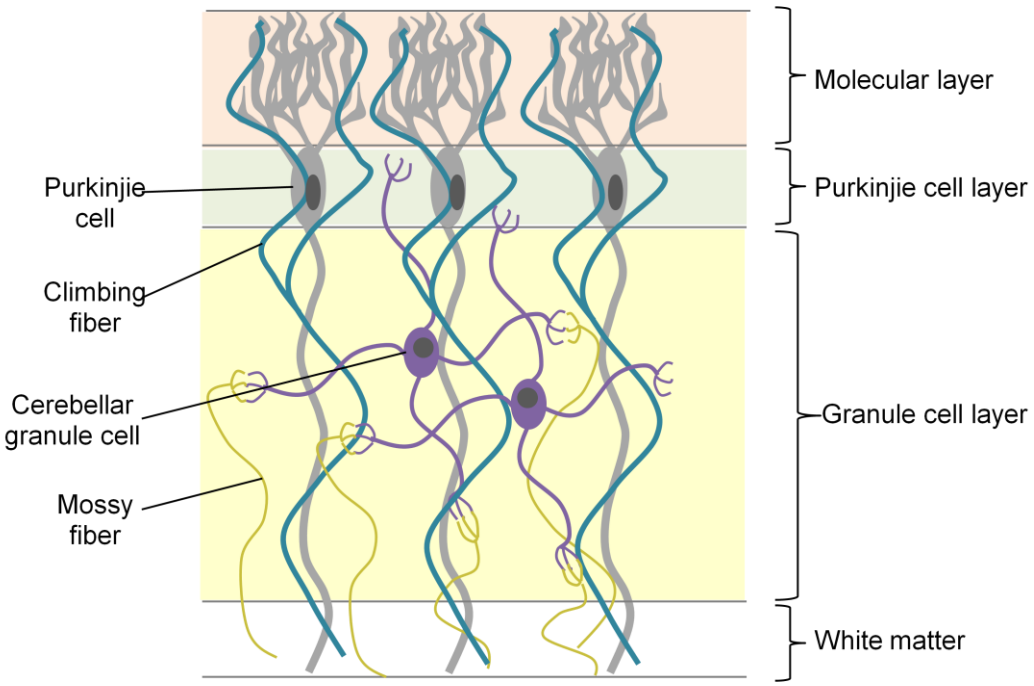

Figure 1.2 Cells and circuitry of the cerebellum. *The cerebellum consists of four distinct layers – the molecular layer into which dendrites of the purkinjie cells project; the purkinjie cell layer, containing the purkinjie cells; the granule layer consisting of cerebellar granule cells; and the while matter, from which excitatory signals originate. Excitatory imputs originate in the white matter from the mossy fibers, which synapse with the cerebellar granule cells. Excitatory signals are propagated into the dendrites of the Purkinjie cells, which process excitatory inputs from the climbing fibers.*

1.1.2 Glia

The CNS consists of four main types of glial cells: astrocytes, oligodendrocytes, ependymal cells and brain macrophages (including microglia), which provide maintenance and trophic support to neurons. Signalling between neurons and glia is bi-directional, and these interactions are important for the functioning of the CNS, with many neurodegenerative diseases arising as a result of the breakdown in these interactions. Glia promote the maintenance, survival, repair, and also death and clearance of damaged neurons both in physiological and pathophysiological conditions.

1.1.2.1 Astrocytes

Astrocytes are the predominant glial cell type in the CNS (He & Sun 2007). There are three astrocytic types, namely radial, fibrous and protoplasmic astrocytes. Radial astrocytes are found radiating from ventricles and at the ventricular zone (VZ), where they are implicated in neuronal pathfinding, and promote sub-ventricular zone (SVZ) stem cell production, that aid regeneration of the adult brain (Doetsch 2003). Fibrous astrocytes are located in the white matter, and their end feet contact the capillaries surrounding the brain, forming the blood brain barrier (BBB), a component of the first line of immune defence which prevents the infiltration of pathogens or other cell types into the CNS (Zlokovic 2008). Protoplasmic astrocytes are located in the grey matter, and their end feet envelop synapses, facilitating synaptic transmission and synaptogenesis (Pfrieger and Barres, 1997).

Astrocytes have numerous roles in synaptic transmission, metabolism and regulation of neuronal interactions. One astrocyte in the adult rodent brain interacts with as many as 10,000 synapses (Bushong et al. 2003; Bushong et al. 2004), and a role for astrocytes in synaptogenesis has been suggested by Pfrieger & Barres (1997), who showed that retinal ganglion neurons (which can survive without glial support) cultured in the absence of astrocytes formed fewer synapses when compared to cultures grown in the presence of astrocytes or in astrocyte conditioned media, therefore suggesting that astrocytes release soluble factors that promote synaptogenesis (Ullian et al. 2004). Furthermore, astrocytes express neurotransmitter receptors and ion channels, which enable them to regulate neuronal activity through their ability to take up neurotransmitters, neuropeptides and ions. This also mediates a change in astrocyte activation state and a reduction in their capacity to buffer potassium in the extracellular space, consequently enhancing neuronal activity (Hatton 2002).

Astrocytes are implicated in neuronal metabolism and energy regulation through the lactate shuttle (Fig. 1.3). Neurons have a preference for lactate as an energy source, which is

produced from glucose in astrocytes in an activity dependant manner (Pellerin et al. 2007). Neuronal glutamate release into the extracellular matrix promotes the activity of astrocytes, resulting in increased lactate production and subsequent availability to neurons (Porras et al. 2008). Furthemore, down-regulation of astrocytic glutamate transporters reduces lactate production, thereby decreasing the energy available to neurons and contributing to the pathology of neurodegenerative conditions (Cholet et al. 2001).

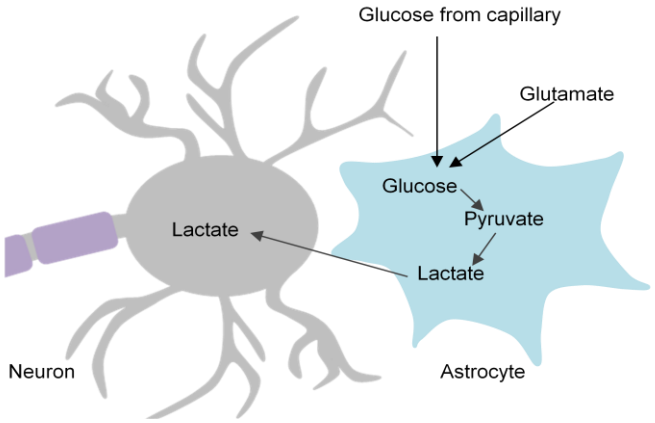

Figure 1.3 The lactate shuttle. *Neurons preferentially use lactate as an energy source, which is produced by astrocytes. Glucose is taken up by astrocytes from the capillaries, which is stimulates glycolysis to promote the production of pyruvate and lactate. In addition, glutamate is taken up by astrocyes through glutamate transporters and is also converted to glucose for use in glycolysis. Lactate is then released from astrocytes and taken up by neurons through monocarboxylate transporters for use as an energy source.*

Neurons and astrocytes interact through gap junctions, and astrocytes represent the largest gap-junction coupled intracellular network within the brain (Nakase & Naus 2004). The communication between neurons and glia through gap junctions allows the co-ordination of intrinsic or elicited metabolic and electrical responses (Nagy et al. 2004), and in this way the breakdown in neuronal glial interactions in neurodegeneration has either a protective or detrimental effect. *In vitro* studies have shown that induction of astrocytic inducible nitric oxide synthase (iNOS) or an increase in intracellular calcium concentration, often associated with cell stress, decreases gap junction communication between neurons and astrocytes,

which protects neurons from cytotoxic signals that require direct cell contact (Bolaños & Medina 1996). However, the reduction in communication between astrocytes and neurons can result in the depletion of neuronal energy and neuronal damage (Salmina 2009).

Changes in the interactions between neurons and astrocytes plays an important role in the progression of neurodegenerative diseases. During neurodegeneration, astrocytes undergo morphological and functional changes, including the up-regulation of glial fibrillary associated protein (GFAP) expression, proliferation, and accumulation at neuronal plaques, which can be protective through their degradation of these plaques, but can also lead to a change in the metabolic state of astrocytes, and a reduction in lactate production, which is detrimental to neuronal survival (Salmina 2009).

1.1.2.2 Oligodendrocytes

The primary function of oligodendrocytes is axonal myelination, which enables the propagation of action potentials (Fig. 1.4). Oligodendrocytes are small, highly branched glial cells, and a single oligodendrocyte can extend its processes to up to 50 axons, ensheathing each axon with 1 mm of myelin (Nave 2010). Myelination is a multistep process, in which oligodendrocyte precursor cells (OPC's) migrate to and make physical contact with an axon, which is mediated by the axonal expression of N-cadherin in the immature CNS, which also facilitates OPC maturation to oligodendrocytes (Schnädelbach et al. 2001). Inhibition of N-cadherin expression attenuates OPC maturation and myelination, and expression of axonal N-cadherin is down-regulated in the mature CNS, suggesting that myelination is a result of early contacts between neurons and oligodendrocytes in the developing brain (Schnädelbach et al. 2001). The critical role of oligodendrocytes in the CNS is demonstrated in MS, in which the myelinating capacity of oligodendrocytes is compromised, and myelin degradation causes neurotoxicity, which, coupled with the inability of mature oligodendrocytes to re-myelinate

axons, leads to an inability of neurons to propagate action potentials, resulting in MS pathology (Fancy et al. 2010).

Oligodendrocytes also regulate extracellular potassium ion concentration, and secrete growth factors, including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), thereby providing trophic support to neurons. Oligodendrocytes are also implicated in the metabolism of cholesterol to pregnalone, a neurosteroid involved in the regulation of neuronal glial interactions (Nichols 1999). Steroidogenesis mediated by oligodendrocytes regulates the production of cytokines, growth factors, nitric oxide (NO), ApoE and heat-shock proteins, which are all important for neuronal survival and degeneration (Garcia-Ovejero et al. 2005). Degradation of oligodendrocytes in diseases such as MS may therefore contribute to the reduction in neuronal glial interactions, which perpetuates neurodegenerative disease.

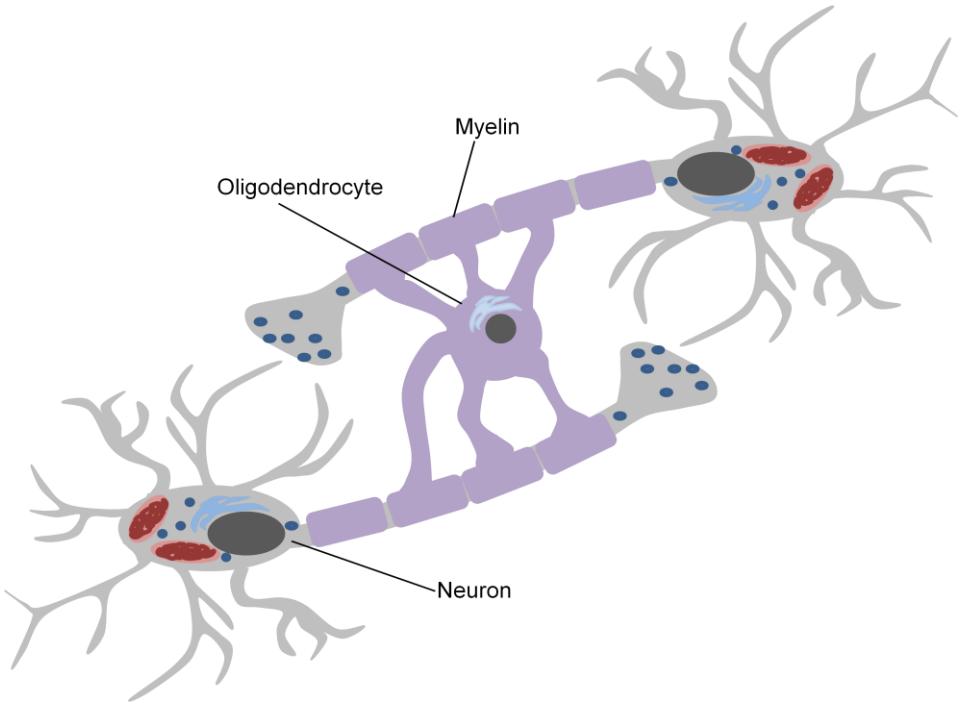

Figure 1.4 Schematic of an oligodendrocye cell myelinating axons. *Oligodendrocytes are highly branched cells that myelinate many neuronal axons. This aids the propagation of the action potential.*

1.1.2.3 Ependymal cells

Ependymal cells are ciliated epithelial glia that line the ventricular surface of the brain and enable the circulation of cerebro-spinal fluid (CSF) around the CNS which facilitates the transport of nutrients, and is implicated in the removal of toxins (Del Bigio 2010). The cilia of ependymal cells beat in a co-ordinated manner, which is facilitated by communications between gap junctions and innervation (Del Bigio 2010). Ependymal cells are typically found at the VZ and SVZ and in this location, they mediate the migration of neural stem cells and organise the SVZ through the production of extracellular matrix and adhesion molecules (Hauwel et al. 2005). Furthermore, ependymal cells facilitate axonal guidance in the CNS during development, and the movement of the cilia also creates concentration gradients of nutrients and growth factors, enabling neuroblast migration (Sawamoto et al. 2006). Ependymal cells also provide trophic support to neurons and other glial cells through the production of growth factors, such as fibroblast growth factor (FGF), which is up-regulated in ependymal cells after ischaemia in rats (Hayamizu et al. 2001).

1.1.2.4 Brain macrophages

Brain macrophages consist of several distinct populations of specialised cells. Macrophages located in the ventricular margins are known as supraependymal cells; epiplexus cells are found in the choroid plexus; subarachnoid macrophages are located in the meninges; and microglia are found in the parenchyma. Brain macrophages therefore cover every area of the brain, where they act as immune cells and survey the CNS for infiltrating pathogens or damaged neurons. They can phagocytose or repair damaged neurons, and also interact with the adaptive immune system to elicit an immune response, thereby enabling the entry of systemic immune cells into the parenchyma. Microglia are the focus of this study and will be discussed in greater detail.

1.2 Microglia in heath and disease

Microglia, the immune-competent cells of the CNS (Färber & Kettenmann 2005), represent 12% of the total cell number in the brain and 20% of glial cells (Block et al. 2007) and can instigate innate immunity within the brain parenchyma following immune challenge (Walter & Neumann 2009). Microglia were first charachterised by Rio Hortega (1932), who recognised focal areas of invasion following brain lesions, termed "fountains of microglia" in the corpus callosum and other white matter areas (reviewed in Färber & Kettenmann 2005), which has lead to further investigations into the protective and immune roles of microglia.

Microglia are derived from myeloid precursors, and appear in the neuroepithelium during early embryonic development (Walter & Neumann 2009) before invasion into the CNS during late embryonic development where they transform into microglia (Ling & Wong 1993). The monocytic origin of microglia was determined in the 1980's in studies showing that ramified microglia express complement and Fc receptors as well as macrophage specific membrane glycoprotein (Perry et al. 1985). Furthermore, (Streit & Kreutzberg 1987) demonstrated that ramified and amoeboid microglia could be visualised by lectin binding, a histochemical marker typically used to label blood monocytes.

Microglia exist in a range of activation states, enabling them to respond appropriately to their environment (Stence et al. 2001) (Fig. 1.5). In the normal brain, microglia have a ramified morphology, and are termed "resting microglia", however in this state, microglia are highly dynamic, enabling them to constantly survey their microenvironment through extension and retraction of their processes (Nimmerjahn et al. 2005; Davalos et al. 2005). In this way, microglia monitor a defined area of the CNS and respond rapidly to any changes in their microenvironment without making physical contact with other cells (Färber & Kettenmann 2005). Resting microglia do not express the full repertoire of immune receptors seen on activated microglia, however, they do express enzymes implicated in the removal of

neurotransmitters from the CSF, and are involved in maintaining neuronal function and structure (Booth & Thomas 1991; Rimaniol et al. 2000).

There has been much debate over the role of microglia in the normal brain under physiological conditions; however, their highly dynamic resting state suggests that their main role is in monitoring the CNS for damage. This suggestion has been supported by *in vivo* imaging of GFP - expressing microglia, which has shown that *in situ*, microglia continuously re-organise their processes, enabling them to probe their environment for activating signals radiating from damaged neurons (Davalos et al. 2005; Fetler & Amigorena 2005). The signals responsible for this continuous remodelling of microglial processes are not well understood (Garden & Möller 2006), although microglia are retained in this ramified state by a range of neuronal interactions.

The interaction between the neuronal membrane glycoprotein CD200 (OX2) and the microglial CD200 receptor (CD200R) regulates microglial activity (Hoek et al. 2000). Microglia are less ramified, aggregated, and express higher levels of immune receptors typically found on activated microglia in CD2000 knockout mice, demonstrating the importance of this interaction in microglial quiescence (Hoek et al. 2000). Microglia are also actively repressed by electrically active neurons, and the removal of this tonic inhibition following neuronal damage leads to microglial activation (Neumann 2001). Two photon microscopy has revealed that microglia are present at, and make contacts with neuronal synapses approximately once an hour, with each contact lasting around 5 min, and microglia make fewer contacts with less active synapses, which promotes microglial activation (Wake et al. 2009). Furthermore, the chemokine fractalkine expressed on neurons also regulates microglial activation, and cleavage of fractalkine from neurons is a microglial activating stimulus which promotes chemotaxis (Chapman et al. 2000). Neurons therefore play an important role in microglial activation state.

Microglia are activated by a variety of stimuli released from damaged or dying neurons, and other cells of the CNS. In response to immunological stimulation, activated microglia exhibit an amoeboid morphology enabling increased mobility to sites of neuronal damage (Fig. 1.5). Microglial activation is a plastic process, with cells changing from a ramified morphology, to a hyper-ramified morphology before finally becoming amoeboid, which facilitates migration to sites of neuronal injury (Raivich 2005). Microglia respond to signals released from damaged neurons, such as adenosine tri-phosphate (ATP) (Booth & Thomas 1991). Microglia fail to respond to CNS injury following blockade of ATP signalling, whilst application of ATP to microglia induces activation to levels seen during CNS injury (Davalos et al. 2005). Furthermore, physical damage to CNS tissue through the formation of lesions or damage to blood vessels or the BBB which results in an influx of immune cells from the periphery, also promotes microglial activation, and the damage of blood vessels specifically may induce this activation through exposure of microglia to blood-derived factors such as thrombin (Möller et al. 2000), albumin or leukocytes (Nimmerjahn et al. 2005).

Activated microglia are characterised by a number of phenotypic and morphological changes. A hallmark of microglial activation is the retraction of processes and enlargement of the cell body and nucleus, characteristic of the amoeboid morphology (Fig. 1.5). Activated microglia also express a number of immune receptors and immunomodulatory proteins such as the major histocompatibilty complex II (MHC II), complement factors, and growth factors (Rimaniol et al. 2000). In addition, activation also promotes the release of mediator substances such as cytotoxic proteases, reactive oxygen species (ROS), reactive nitrogen species (RNS) and glutamate (reviewed in Block et al. 2007). Microglial activation also correlates with proliferation and recruitment of immune cells to the site of neuronal injury, and activated microglia also exhibit a phagocytic phenotype (Ling & Wong 1993).

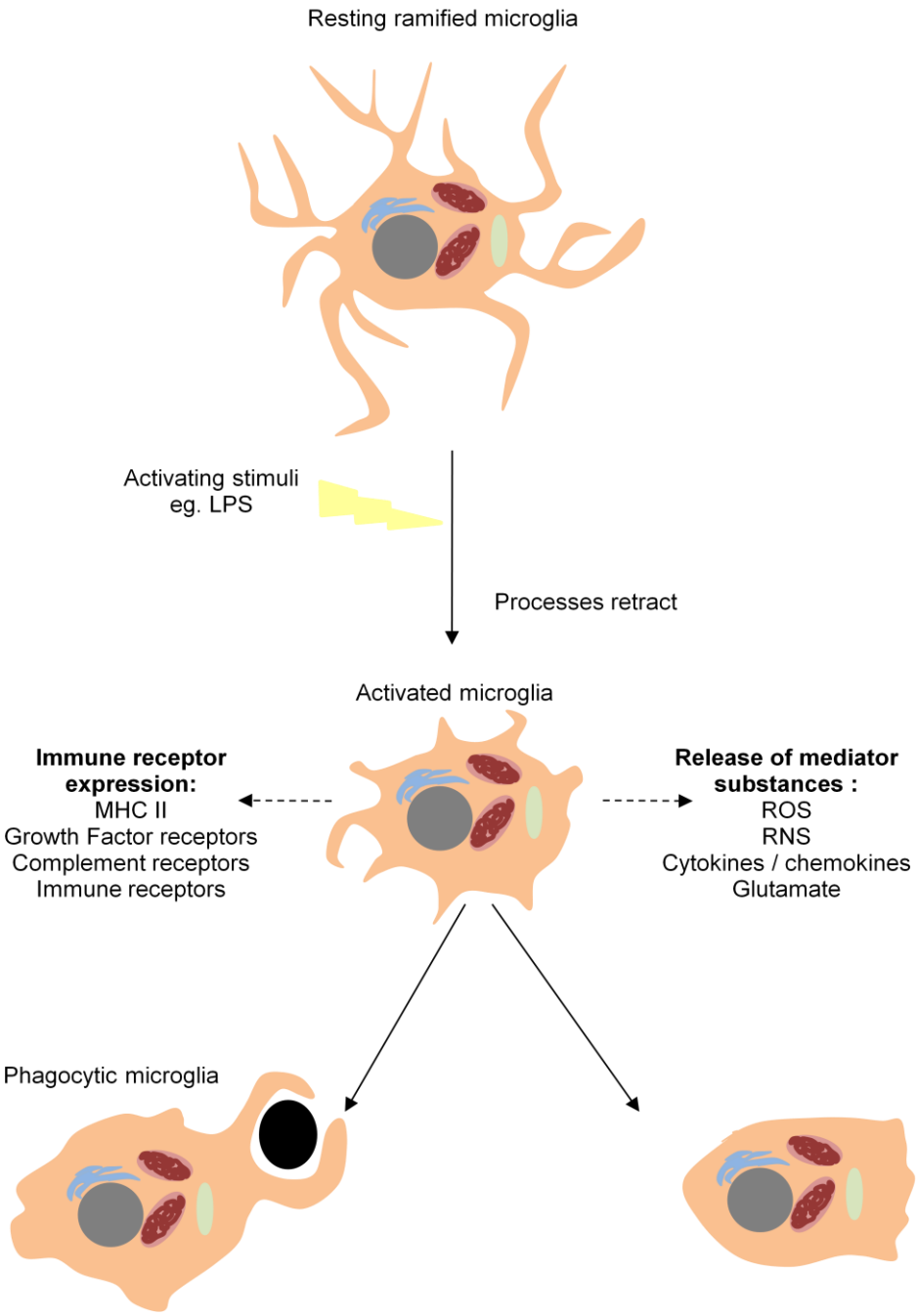

Figure 1.5 Microglial activation states. *Microglia are found in a resting, highly ramified state where they extend and retract their processes in response to changes in the microenvironment. Activation induces a retraction of processes and the expression of immune receptors and enhances the antigen presenting properties of microglia, and also induces the release of mediator substances. Activated microglia can become migratory and move to the sites of neuronal injury, or phagocytic, enabling them to engulf pathogens and toxic proteins.*

Microglial activation can be either protective or toxic (Streit 2002). Activated microglia can maintain and support neuronal survival by releasing trophic and anti-inflammatory molecules, such as transforming growth factor-β (TGF-β) and interleukin-6 (IL-6) (Boche et al. 2006); removing invading pathogens or toxic products (Liu et al. 2002), and facilitating the guidance of stem cells to lesion sites to promote neurogenesis (Walton et al. 2006). However, microglial activation can also release cytotoxic substances such as NO or peroxynitrite (Colton & Gilbert 1987), superoxide (Chéret et al. 2008), and pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (Bruce et al. 1996). Furthermore, microglial activation can be perpetuated by autocrine pathways, in which cytokines released from microglia may enhance microglial activation (Walter & Neumann 2009).

1.3 Micrioglial activation in neurodegeneration

Microglial activation is a hallmark of several neurodegenerative diseases, and microglial reactivity can perpetuate neurodegeneration. In neurodegenerative diseases such as AD, PD, MS and ischaemia, microglia are activated by neurotoxic peptides or blood borne proteins. Microglial activation is a characteristic of AD pathology, in which the beta-amyloid (Aβ) peptide (which aggregates to form neurotoxic plaques) promotes a reactive phenotype (Rogers et al. 2002). This activation can be both protective and toxic, as Aβ mediates microglial phagocytosis of Aβ plaques through activation of complement cascades (Rogers et al. 2002), which is initially seen as a protective mechanism to prevent further neuronal damage (Mandrekar-Colucci & Landreth 2010), however, this rapidly induces a toxic phenotype, inducing microglial proliferation and the release of cytokines such as TNF-α and IL-1β as the disease progresses and Aβ load increases (Jekabsone et al. 2006). There is evidence to suggest that microglial activation by Aβ is mediated through its interaction with

scavenger receptors (SR) (Husemann et al. 2002), and that this promotes a phagocytic response (El Khoury et al. 1996). The involvement of microglial SR in AD has been further studied by (Hooper et al. 2009), who show that chromogranin A (CgA), which is highly expressed and released in neurites containing Aβ plaques (Pocock & Liddle 2001), activates microglia through SR, and increases iNOS expression which is a hallmark of microglial activation. Microglia may also be activated in AD through an interaction of Aβ with receptor for advanced glycosylation end products (RAGE), which induces the release of ROS and neuronal death (Yan et al. 1996); and microglia are also activated by Aβ binding to the Serpin-enzyme complex receptor, which may be neuroprotective through clearance of Aβ monomers (Boland et al. 1996).

Activated microglia are found in the substantia nigra (SN) of patients with PD, where they release high levels of superoxide, which contributes to the loss of dopaminergic neurons (Gao et al. 2002). Microglia activated in this way also release TNF-α, IL-1β and interferon-γ (IFN-γ) (Hirsch et al. 1998). A pathological hallmark of PD is α-synuclein, which is found in aggregates in PD brains, and these aggregates are surrounded by activated microglia which release superoxide resulting in dopaminergic neuron toxicity seen in PD (Zhang et al. 2005). Excess neuromelanin, which is found in dopaminergic neurons, is also a hallmark of juvenile and idiopathic PD, also activates microglia. *In vitro* studies have shown that microglia treated with neuromelanin from PD brains expressed and released TNF-α, IL-6 and NO, which mediate PD pathogenesis (Wilms et al. 2003).

Activated microglia are also associated with MS lesions, in which the BBB breakdown and presence of myelin debris enhances microglial activation and induces the phagocytosis of oligodendrocytes and myelin, which contributes to axonal degeneration (Muzio et al. 2007). However, whether these activated microglia contribute to disease progression or facilitate re-myelination is unclear, as some studies suggest that myelination is increased in the presence

of microglia activated by zymosan, a potent microglial activator (Setzu et al. 2006) which increases BDNF expression thereby inducing oligodendrocyte recruitment to lesion sites (Stadelmann et al. 2002).

Microglia are therefore implicated in both the maintenance of the CNS and also the course of neurodegenerative diseases. This dual effect makes microglia an attractive target for therapeutic intervention in diseases in which microglia contribute to disease progression, as harnessing their neuroprotective capacity could slow neurodegeneration, and may facilitate neuronal repair. Their interaction with the immune system also has important ramifications for diseases with a systemic element, making them important targets for future therapies.

1.4 The immune system

There are two branches of the immune system, the innate and adaptive responses (Roit et al 1987). The innate immune system is the first line of defence against external pathogens and infection, and is composed of phagocytes such as macrophages, neutrophils, natural killer cells and soluble factors such as lysozyme and complement proteins. Activation of the innate immune system induces cytokine production, which recruit cells of the adaptive immune system to the site of injury (Roit et al 1987). The adaptive immune system forms the second line of defence, and is composed of B and T cells, which enable the body to mount a long lasting immune response to provide long-lived specific immunity (Roit et al 1987). Macrophages, such as microglia, link the innate and adaptive immune responses through the expression of MHC II, which enables the presentation of antigen to T cells of the adaptive immune system, which then promotes antibody production by B cells.

1.4.1 The brain as an immune privileged organ

The brain has long been considered an immunologically privileged site due to the inaccessibility of circulating immune cells to the CNS. This immune privilege is

demonstrated by studies showing that tissue grafts into the brain are not rejected by the host (Head & Griffin 1985). Furthermore, the brain also fails to mount an immune response upon injection with mycobacterium (which induces an aggressive immune response in other organs) (Matyszak & Perry 1998). However, this view has been challenged in recent years, and there is now an increasing focus on the brain as an immune-competent organ (Xiao & Link 1998). Research shows that brain immunity is a collection of CNS driven mechanisms that actively regulate T cell responses within the CNS parenchyma. The CNS can shape immune responses by: allowing activated T cells to traffic through the CNS for surveillance (Cserr & Knopf 1992); the presence of a lymph- like system (Ransohoff et al. 2003); and the activation of endogenous glial cells which participate in innate immune responses (Owens et al. 1994). In this way, the brain is now considered to be immunologically active, enabling a variety of responses to aid protection against several inflammatory and neurodegenerative diseases.

The brain is separated from the immune system by the BBB, a physical barrier formed of endothelial cells and astrocytes which prevents the entry of migratory cells and other pathogens to the CNS parenchyma (Ransohoff et al. 2003) (Fig. 1.6). The endothelial tight junctions of the BBB prevent the movement of leukocytes across the epithelium, aiding the immunological isolation of the brain. Furthermore, the BBB prevents the movement of solutes into the CNS parenchyma (Ransohoff et al. 2003).

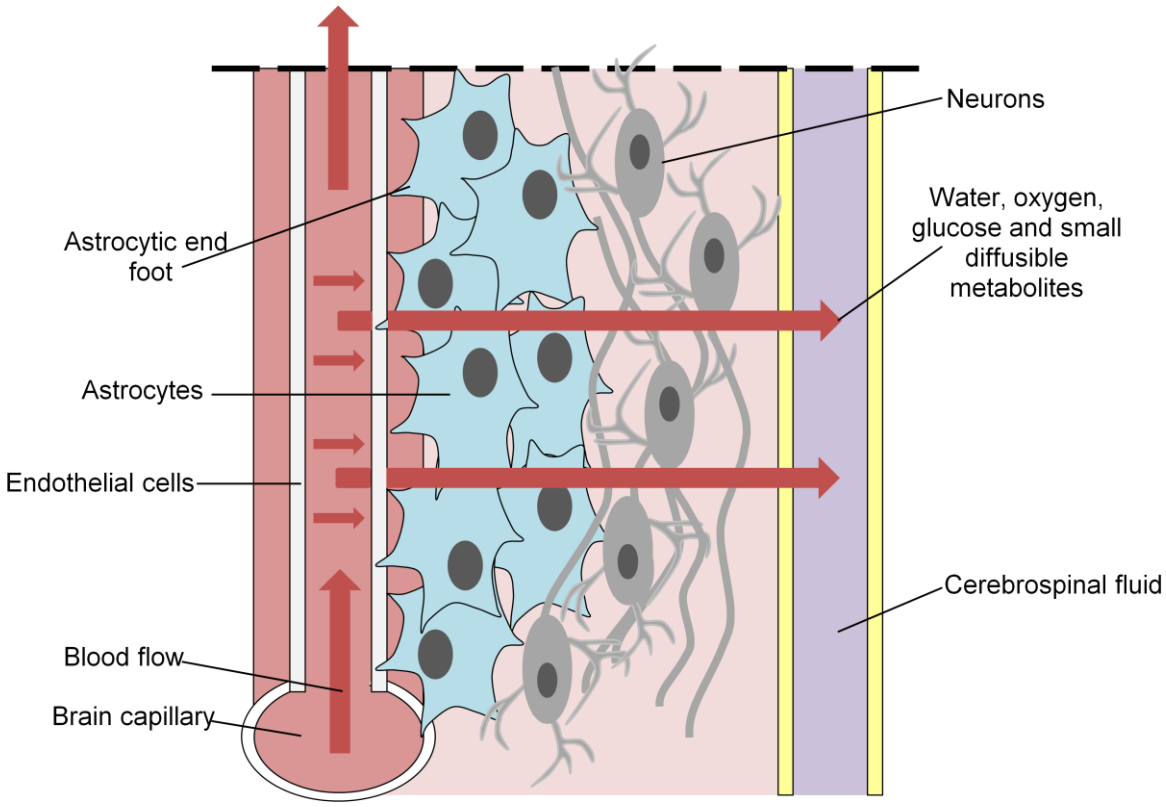

Figure 1.6 The blood brain barrier. *The blood brain barrier (BBB) is a physical barrier to prevent the influx of toxins and pathogens into the brain parenchyma. It is composed of endothelial cells, contacted by astrocytic end feet, which form tight junctions that act as a barrier. The BBB allows water, oxygen, glucose and small diffusible metabolites to pass through to the CSF, however astrocytes can sense pathogens and a breakdown in endothelial cell integrity after infection, which enables the recruitment of microglia and release of immune mediators.*

The BBB has a regulatory role in the development of an immune response in the CNS. Injury to the CNS activates endothelial cells and astrocytes (attached to the BBB via the astrocytic end foot), which compromises the integrity of the tight junctions allowing leukocytes to enter the brain, where they then mount an immune response promoting inflammation (Wilson et al. 2010). The cells of the BBB also secrete cytokines and express adhesion molecules, which aid the adhesion and penetration of circulating immune cells (Xiao & Link 1998). Regulation of the BBB and the loss of integrity of this physical barrier to the CNS therefore promotes

other immune and inflammatory responses in the CNS, seen in several neuroinflammatory and neurodegenerative conditions.

The migration of immune cells such as T cells, macrophages and leukocytes is up regulated during the inflammatory response in the CNS, which can be caused by a variety of factors, such as the Aβ plaques found in AD, and myelin deposits found in MS, in addition to other neuroinflammatory mediators, such as oxygen-glucose deprivation seen in ischaemia (Carson et al. 2006). The infiltration of these circulating immune cells into the CNS is a key event in the progression of neuroinflammatory conditions.

The interaction between the nervous and immune systems underlies the regulation of immune responses in the CNS. There is a bi-directional communication between both systems that modulates the immunological activity of the CNS (Haddad 2008), which is mediated by soluble factors such as cytokines, immune cells and endogenous glial cells that have immune functions. Cytokines are secreted both by peripheral immune cells and also by astrocytes and microglia within the CNS, and in this way, the immune system can direct the CNS to mount an immune response, by eliciting the activation of the endogenous immune cells (microglia); or the CNS can induce a further immune response, by cytokines promoting a disruption of the BBB, thus enabling the infiltration of T cells and B cells into the CNS parenchyma (Haddad 2008). Neurons themselves also regulate brain immunity by maintaining microglia in a quiescent state (Neumann 2001) as discussed previously. Neuronal activity regulates microglial MHC expression, and facilitates the presentation of antigen and the subsequent recruitment of T cells to the brain parenchyma. Signals from damaged neurons are key mediators of the microglial immune response, and are also implicated in the cross talk between the nervous and immune systems.

There is an important interplay between the nervous and immune systems, and there is evidence to suggest that the CNS regulates its own immune response. In light of this research, the roles of immune cells both within the CNS and also those infiltrating the CNS parenchyma are becoming a focus of investigation in neurodegenerative disease, however, here the roles of the endogenous CNS immune cells, the microglia, and the production of superoxide as an immune and regulatory mediator is the focus of this research.

1.5 Reactive oxygen species in the central nervous system

ROS include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$) (Kamata & Hirata 1999), which are produced in neurons and glia by a number of enzymes. Microglia produce large amounts of ROS when activated with neurotoxic peptides such as Aβ (Wilkinson et al. 2006), myelin, and PD toxins (Gao et al. 2003), therefore microglial ROS production is an important hallmark of neuroinflammatory diseases.

Mitochondria are an important source of ROS, which is produced as a by-product of energy metabolism. During ATP production, oxygen accepts a hydrogen ion to produce H_2O; however, a small percentage (<5%) of electrons are leaked (Halliwell, 2001), which leads to the production of superoxide. The endoplasmic reticulum (ER) is another site of ROS production, where superoxide is generated by the leakage of electrons from the cytochrome p450 reductase (Cross & Jones 1991). Furthermore, superoxide is also produced by the hypoxanthine / xanthine oxidase, lipoxygenase, cyclooxygenase, and the nicotine adenine dinucleotide phosphate (NADPH) oxidase (Kamata & Hirata 1999), which is the focus of this thesis. To counteract the effects of ROS and to maintain the redox state of the cell, cells produce anti-oxidants such as glutathione (GSH), which converts hydrogen peroxide to water in conjunction with glutathione peroxidase; superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide then water; catalase, which converts hydrogen peroxide to

water; and thioredoxin (TRX), which has a redox sensitive dithiol / disulphide that reduces hydrogen peroxide and refolds oxidised proteins (reviewed in Kamata & Hirata 1999). The maintenance of cellular redox balance is important for cell health and survival, as increased ROS production can lead to pathology or disease progression in several neurodegenerative disorders.

1.5.1 Pathology and physiology of ROS and the immune response

ROS are essential for the activation of signalling pathways, cellular processes and gene regulation (Allen & Tresini 2000). The cellular responses elicited by ROS typically modulate cytokine, growth factor or hormone action and secretion; ion transport; transcription; neuromodulation; or apoptosis (Allen & Tresini 2000). In this way, physiological levels of ROS maintain the cell homeostasis, whilst during pathology, ROS levels are often increased, elevating the expression of apoptotic genes and inflammatory mediators, resulting in inflammation, cell death, and disease progression.

Many proteins implicated in cell survival and signalling are modulated by ROS generated under physiological conditions. The main target of redox regulation on proteins is a sulphydryl group (RSH) on cystine residues, which can be oxidised to a disulphide bond (RSSR), sulphenic acid (RSOH), sulphinic acid (RSO_2H), or sulphonic acid (RSO_3H) (Kamata & Hirata 1999). Stimulation of cells with ROS often activates signalling cascades that are typically activated by growth factors. For example, mitogen activated protein kinase (MAPK), protein kinase C (PKC), phospholipase C (PLC), phosphatidyl inositol-3 kinase (PI3-K), c-Jun N-terminal kinase (JNK) and intracellular calcium levels are all modulated by ROS (Kamata & Hirata 1999). ROS regulation of cell signalling is important for cell survival. For example, hydrogen peroxide promotes Akt phosphorylation through its stimulation of the PI3-K pathway (Konishi et al. 1997). In addition, ROS play an important role in lipid metabolism, and promote tyrosine phosphorylation on PLCγ, which can hydrolyse

phosphatidylinositol 4,5-bisphosphate (PIP_2) into Inositol (1,3,5) triphosphate ($Ins(1,4,5)P_3$), which mobilises intracellular calcium from the ER (Schieven et al. 1993); and also promotes the production of diacylglycerol (DAG), which activates PKC (Qin et al. 1995). Both calcium and PKC are second messengers used in a variety of pathways, in particular those involved in synaptic plasticity and long term potentiation (LTP) (Berridge 1993). ROS are also essential for the activity of transcription factors, which are typically implicated in cell surivial or apoptosis (Kamata & Hirata 1999).

Growth factor or cytokine binding to receptors initiates ROS generation, and inhibition of ROS production following ligand binding attenuates signalling pathways and cellular responses expected from receptor activation (Finkel 2000). In vascular smooth muscle cells (VSMC's), catalase overexpression inhibits the ability of platelet derived growth factor (PDGF) to phosphorylate tyrosine residues on a number of effector proteins (Sundaresan et al. 1995). However, ROS production from such receptor signalling can be detrimental as well as protective, and elevated ROS production is a hallmark of many neurodegenerative diseases.

ROS are implicated in pathology, and the over-production of ROS, or stimulation through overactivation / inhibition of receptors can induce the release of toxic factors and death in several cell types. Exposure of cells to inflammatory cytokines or pathogens can enhance the expression and activity of ROS producing enzymes, resulting in a redox disequilibrium, and oxidative damage. An increase in ROS is seen in AD and PD. In PD, the levels of cytokines such as TNFα, IL-1β and IFNγ are increased in the SN (Sawada et al. 2006). It is thought that these cytokines are originally released from activated microglia and that they may contribute microglial ROS production through autocrine and paracrine feedback loops, as stimulation of microglia *in vitro* with these cytokines alone promotes superoxide production through activation of the NADPH oxidase, along with expression of iNOS and subsequent NO

production, which in combination form peroxynitrite, a potent neurotoxin (Brown & Bal-Price 2003; Brown & Neher 2010). Furthermore, the presence of aggregated α-synuclein and lewy bodies characteristic of PD promote microglial activation, and subsequent phagocytosis of these aggregates by microglia can induce ROS production (Thomas et al. 2007).

Microglial ROS production is also implicated in AD progression. Aβ activates microglia, and induces superoxide production through NADPH oxidase activation (Brown & Neher 2010). In addition, Aβ also promotes microglial TNFα which feeds back onto microglial TNFα receptors to enhance microglial activation and ROS production (Meda et al. 1995). Activation of microglial NADPH oxidase following exposure to pathological substances such as Aβ, α-synuclein, neurotoxins and cytokines, and subsequent superoxide production also promotes the activation of intracellular signalling cascades, such as PKC (Konishi et al. 1997) and NF-κB (Schreck et al. 1991) which mediates the expression of pro-inflammatory genes, exacerbating disease pathology. In this way microglial derived intracellular ROS activate microglia and enhance pro-inflammatory signalling (Block 2008). In addition, the Nef protein of the HIV virus promotes microglial superoxide production which leads to the activation of intracellular signalling pathways and subsequent morphological changes consistent with an activated microglial phenotype (Sawada et al. 2006). Intracellular superoxide production therefore regulates microglial activation state and their neurotoxic or neuroprotective roles in pathology (Block 2008).

1.5.2 Production of superoxide by the NADPH oxidase

The NADPH oxidase (EC number 1.6.3.1) is a multi-subunit enzyme that produces superoxide through the oxidation of NADPH to reduced nicotine adenine dinucleotide phosphate (NADP) and hydrogen (H^+) (Fig. 1.8) (Babior 2000). The NADPH oxidase was first identified in phagocytes, which exhibit increased oxygen consumption during phagocytosis, and produce an oxidative burst essential for defence against invading

pathogens (Someya et al. 1999; Sheppard et al. 2005; Mander & Brown 2005). NADPH oxidase expression has since been shown in microglia where it is implicated in the production of intracellular (Chéret et al. 2008; Harrigan et al. 2008) and released (Wilkinson et al. 2006) superoxide. The structure of the NADPH oxidase (Fig. 1.7) was first elucidated in polymorphonuclear neutrophils (PMNs), and it is composed of both cytoplasmic and membrane bound subunits (reviewed in Baboir 2000). The membrane associated subunits form the catalytic domain of the enzyme, which is composed of the gp91phox and p22phox subunits, a flavin adenine dinucleotide (FAD) which is the NADPH binding site (Segal et al. 1992), and two haeme groups, one of which binds gp91phox and the other p22phox (Quinn et al. 1992). Together, these form the cytochrome b_{558} subunit, which facilitates the transfer of electrons from the NADPH oxidase to the membrane. The FAD and haeme groups of the catalytic domain enable electron transfer, and thereby act as a redox pathway (Sheppard et al. 2005). Studies using FAD deficient NADPH oxidase have revealed that this component is the electron carrying intermediate, as removal of this domain inhibits oxidase activity, which could also be mimicked by flavin antagonists (Light et al. 1981). The p22phox and gp91phox interact through their proline-rich and Src homology 3 (SH3) domains, and gp91phox is a H^+ channel, which regulates steady-state FAD reduction (Henderson et al. 1988). Furthermore, truncation of the C-terminal of the gp91phox abolishes NADPH oxidase activity, suggesting the importance of this subunit in enzymatic activity (Burritt et al. 2003).

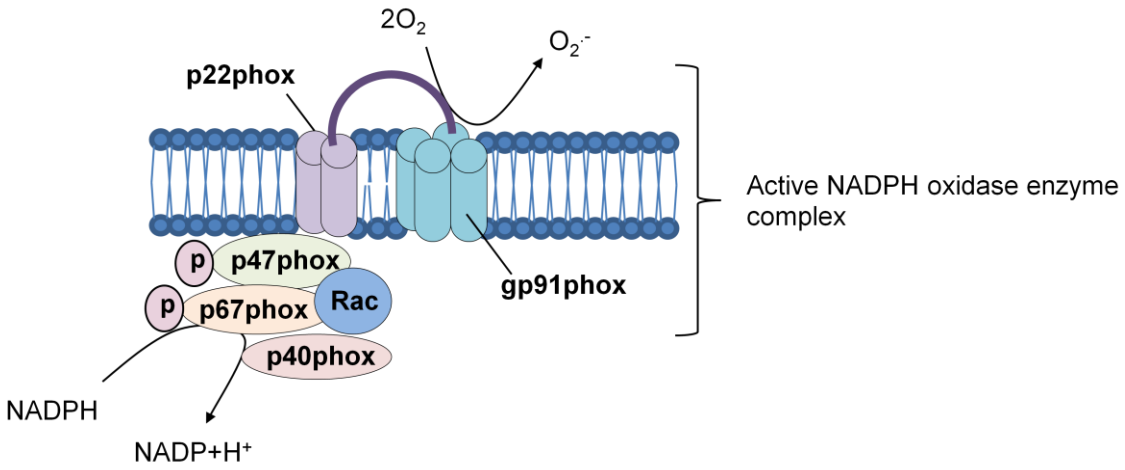

Figure 1.7 Superoxide production from the NADPH oxidase. *The NADPH oxidase is a multi- subunit enzyme composed of the membrane bound gp91phox and p22phox subunits and the cytoplasmic p47phox, p67phox and p40phox subunits. The p47phox and p67phox subunits are phosphorylated to promote association with the membrane bound subunits, which is also facilitated by a Rac GTPase. Upon enzyme assembly, the substrate NADPH is reduced to NADP + H^+ which enables the production of superoxide from molecular oxygen.*

The NADPH oxidase is activated upon the association of cytoplasmic subunits to the catalytic membrane bound domain (Fig. 1.8). The three catalytic subunits of the classical NADPH oxidase are: p47phox, p67phox and p40phox, which are translocated to the membrane bound domains through the activity of a Rac GTPase (Sheppard et al. 2005).

Phosphorylation of the p47phox and p67phox subunits promotes their translocation to the catalytic membrane bound subunit. The p47phox subunit contains an N-terminal phox (PX) domain, which targets the p47phox to PIP_3 in the membrane and the membrane bound subunits (Kanai et al. 2001; Zhan et al. 2002). Furthermore, phosphorylation of the p47phox subunit allows direct association with p22phox, facilitating the binding of p67phox and p40phox to the cytochrome b_{558} (Dang et al. 2001). The C-terminus of p47phox contains serine phosphorylation sites, which are targets for phosphorylation by PKC and MAPK (Yamamori et al. 2000). The p67phox is also essential for catalytic activity of the NADPH

oxidase. It contains a catalytic NADPH binding site for the transfer of electrons to FAD (Cross & Curnutte 1995). Moreover, phosphorylation of p67phox is essential for translocation of the p40phox to the membrane bound subunits (Dusi et al. 1996). The p40phox contains a PX domain which is targeted to PIP_3 in the membrane and is also phosphorylated by PI3-K (Ellson et al. 2006) and PKC at the N-terminal domain. The onset and level of p40phox phosphorylation affects the level of superoxide production (Fuchs et al. 1997), and p40phox can down-regulate oxidase activity by competing with the SH3 domain interactions between the catalytic subunits (Sathyamoorthy et al. 1997).

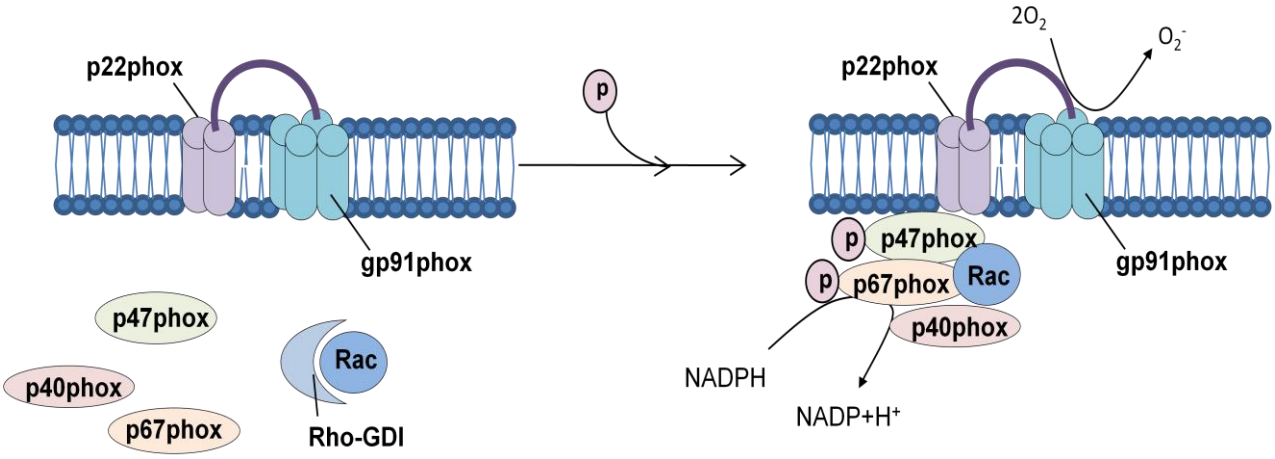

Figure 1.8 Assembly of the NADPH oxidase. *The cytoplasmic subunits of the NADPH oxidase – the p47phox and p67phox subunits are phosphorylated which promotes the recruitment of the p40phox subunit to the p47phox subunit. The three cytoplasmic subunits are then translocated to the membrane bound subunits by a Rac GTPase. This then enables the production of superoxide through the reduction of NADPH to NADP + hydrogen.*

1.5.3 NADPH oxidase isoforms

Different cell types express different isoforms of the NADPH oxidase, which each have a distinct role in the production of intracellular or released superoxide. Many non-phagocytic cells express the p22phox subunit, however of those studied, many did not express the classical gp91phox subunit (Nox2), but instead expressed gp91phox homologues, allowing the expression of functional isoforms of the NADPH oxidase. Furthermore, these isoforms are also comprised of different components of the p47phox, p40phox and p67phox cytoplasmic subunits, which form the classical Nox2 isoform. These different NADPH oxidase isofoms therefore mediate different activities in the cell.

Human cells express seven gp91phox homologues, or Nox proteins, which are found mainly in non-phagocytic cells (Bokoch & Knaus 2003). These are designated Nox1-5, with Nox1 and Nox4 being structurally similar to Nox2, the classical NADPH oxidase described above (Cheng et al. 2001). Nox isoforms have divergent roles during development and ageing, and are expressed in different cells at different developmental stage. Nox2 is expressed in phagocytic cells and is responsible for neutralizing invading pathogens (Baboir 2000). Nox1 is predominantly expressed in colon endothelial cells and VSMC's (Suh et al. 1999), Nox3 is found in fetal kidney cells (Kikuchi et al. 2000) and in the inner ear in adults (Bánfi et al. 2004). Nox4 is predominantly found in the kidney (Shiose et al. 2001), whilst Nox5 is expressed in the spleen (Cheng et al. 2001). The remaining NADPH oxidase isoforms are designated DUOX1 and DUOX2, which are found in the thyroid, and are implicated in host defense (Katsuyama 2010). Nox isoforms are also found in the CNS, which will be discussed in greater detail.

There are many signalling cascades that lead to the activation of different Nox isoforms, which can phosphorylate the different cytoplasmic subunits associated with each Nox

isoform. The focus here will be on Nox1, 2, and 4, which are central to this thesis. The Nox1 and Nox2 isoforms can be independently regulated by different signalling molecules (Chéret et al. 2008), which is due to their different cytoplasmic components (Fig. 1.9). Nox2 can be independently activated by phosphorylation of the p40phox subunit via activation of the PI3-K signalling pathway, as demonstrated in a lymphoma cell line (Ha & Lee 2004), whereas the Nox1 isoform lacks the p40phox subunit, and is preferentially activated by the phosphorylation of the p47phox subunit through a PKCδ dependent mechanism (Seshiah et al. 2002). Nox4 differs from Nox1 and Nox2 as it lacks the cytoplasmic subunits (Fig. 1.9), and is composed only of the membrane bound catalytic domains and its activity is therefore regulated at the transcriptional level (Ellmark et al. 2005). Nox4 activity can however be increased in smooth muscle cells via janus kinase / signal transducer and activator of transcripton (JAK/STAT) signalling and subsequent transcription of the isoform, which contains an interferon stimulated response element (ISRE) or interferon activated site (GAS) element in its promoter region (Manea et al. 2010). Furthermore, studies in endothelial cells deprived of serum in the growth medium have shown that Nox4 is also regulated at the translational level through p38MAP kinase (p38MAPK) signalling, therefore suggesting that the intracellular ROS produced from this isoform may aid cell survival (Peshavariya et al. 2009). Activation of different isoforms of the NADPH oxidase is therefore dependent on different activating stimuli.

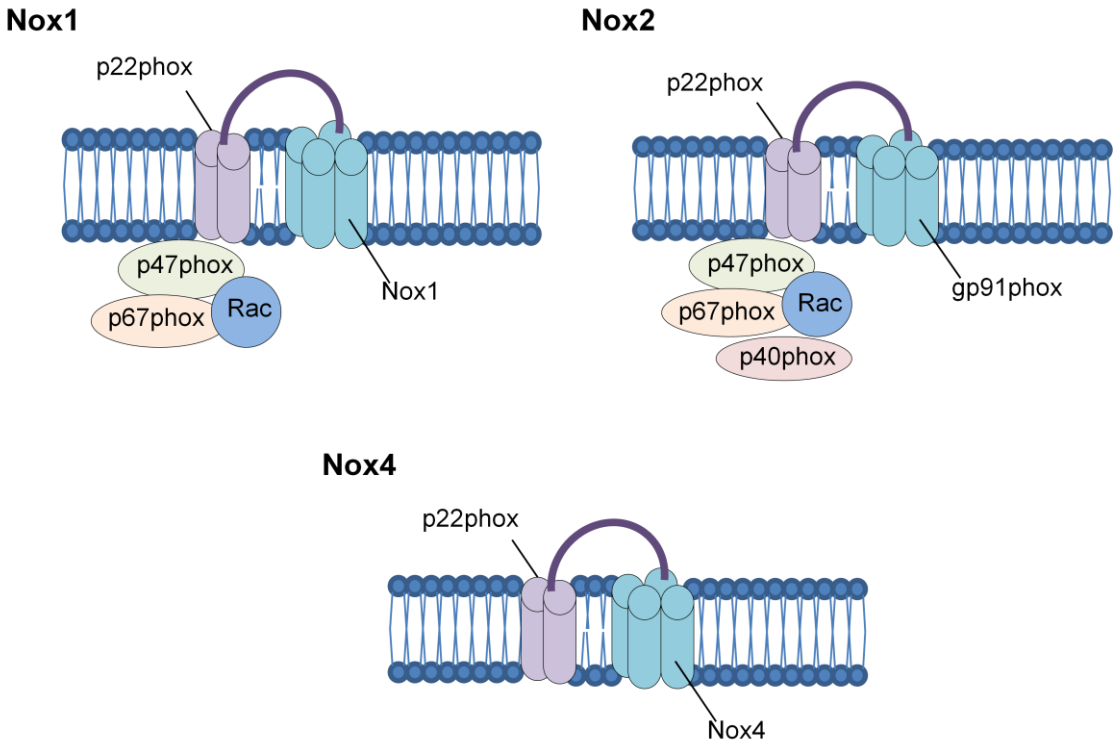

Figure 1.9 The structure of Nox1, Nox2 and Nox2. *Nox1 is comprised of the Nox1 gp91phox homologue which associates with the p22phox membrane bound subunit. Nox1activity is also dependent on the association of the p47phox and p67phoxsubunits to the membrane. The classical NADPH oxidase is Nox2, comprised of the p22phocx, gp91phox, p47phox, p67phox, and p40phox subunits. Nox4 is comprised of membrane bound subunits only, namely the p22phox and Nox4 gp91phox homologue.*

1.5.4 NADPH oxidase in the CNS

Three Nox isoforms – Nox1, Nox2 and Nox4 are expressed in the CNS. Nox2 is expressed in microglia, where it is implicated in the inflammatory response (Infanger et al. 2006), and also in astrocytes, where it is implicated in superoxide production in response to the HIV Tat protein (Song et al. 2010). Nox1 is expressed in human fetal brain tissue (Infangeret al. 2006), and in microglia (Chéret et al. 2008), whilst Nox4 is localized to cortical neurons and capillaries after ischaemia (Vallet et al. 2005), and has recently been identified in microglia, where it promotes glutamate release (Harrigan et al. 2008). In addition to their expression in different cell types, the Nox1, 2 and 4 isoforms also have distinct sub-cellular localizations,

which regulates the redox environment in different organelles or intracellular vesicles. Nox1 has an intracellular localisation and is found on the nuclear membrane in keratinocytes (Chamulitrat et al. 2003) and on endosomal membranes in vascular cells (Miller et al. 2007), and Nox1 is localised to lysosomes in microglia (Chéret et al. 2008). Nox2, the classical NADPH oxidase is an inducible isoform, and is localised predominantly to intracellular phagosomes and at the plasma membrane in microglia (Chéret et al. 2008); in addition, Nox2 is localised to endosomes, specifically those responsible for early receptor mediated signalling, called redoxisomes (Oakley et al. 2009). Harrigan et al., (2008) have shown that microglia express Nox4, however its function and subcellular localisation are not well characterised, but it is believed to be implicated in intracellular H_2O_2 production and is localised at the ER in other cell types, where it provides the correct redox environment for protein folding (Ambasta et al. 2004). The role of these NADPH oxidase isoforms is under investigation in the CNS, and the effects of microglial expression of Nox isoforms will be discussed in greater detail.

1.5.4.1 Microglial expression of the NADPH oxidase

The expression of Nox1, Nox2 and Nox4 (Fig. 1.9) in microglia have distinct physiological and pathophysiological roles (Wilkinson & Landreth 2006; Chéret et al. 2008; Harrigan et al. 2008). ROS, including superoxide, are required to maintain the redox state of microglia, and in this way the NADPH oxidase is essential for the maintenance of this environment. However, in certain circumstances, microglia can become activated or exposed to stimuli that cause excessive ROS production following activation of the NADPH oxidase, which can be detrimental to both the microglia themselves and surrounding neurons and glia. In this way, activation of the microglial NADPH oxidase can be damaging to the CNS and is implicated in pathology associated with several neurodegenerative diseases.

1.5.4.1.1 Microglial NADPH oxidase in physiological conditions

Each NADPH oxidase isoform has a distinct cellular role. Nox1 is responsible for the production of intracellular superoxide, and as such is implicated in cell signalling cascades. Activation of microglial Nox1 induces iNOS and IL-1β expression (Fig. 1.10), and is neurotoxic (Chéret et al. 2008). In addition to being one of the key enzymes responsible for the production of ROS in the oxidative burst, Nox2 also mediates intracellular signalling events, such as those involved in cytoskeletal rearrangements implicated in cell migration (Yan et al. 1995). Furthermore, Nox2 also mediates TNF receptor associated factor 2 (TRAF2) binding to the TNF-receptor 1 / TNF receptor associated death domain (TNFR1/TRADD) complex, promoting NF-κB translocation and cell death (Shen et al. 2004). Nox2 derived ROS in microglia promote microglial activation and proliferation (Jekabsone et al. 2006), release of neurotransmitters (Barger et al. 2007), and also neurotoxicity through the oxidative burst in conditions such as stroke (Block et al. 2007) (Fig. 1.10). Activation of microglial Nox4 is implicated in the release of glutamate through volume regulated anion channels (VRAC) in microglia (Harrigan et al. 2008), and also promotes IL-6 expression in human microglial cells (Li et al. 2010). The main role of the microglial NADPH oxidase however appears to be in pathology, in which the production of ROS are implicated in either neuronal survival or neurotoxicity, depending on the activating stimuli and Nox isoform activated.

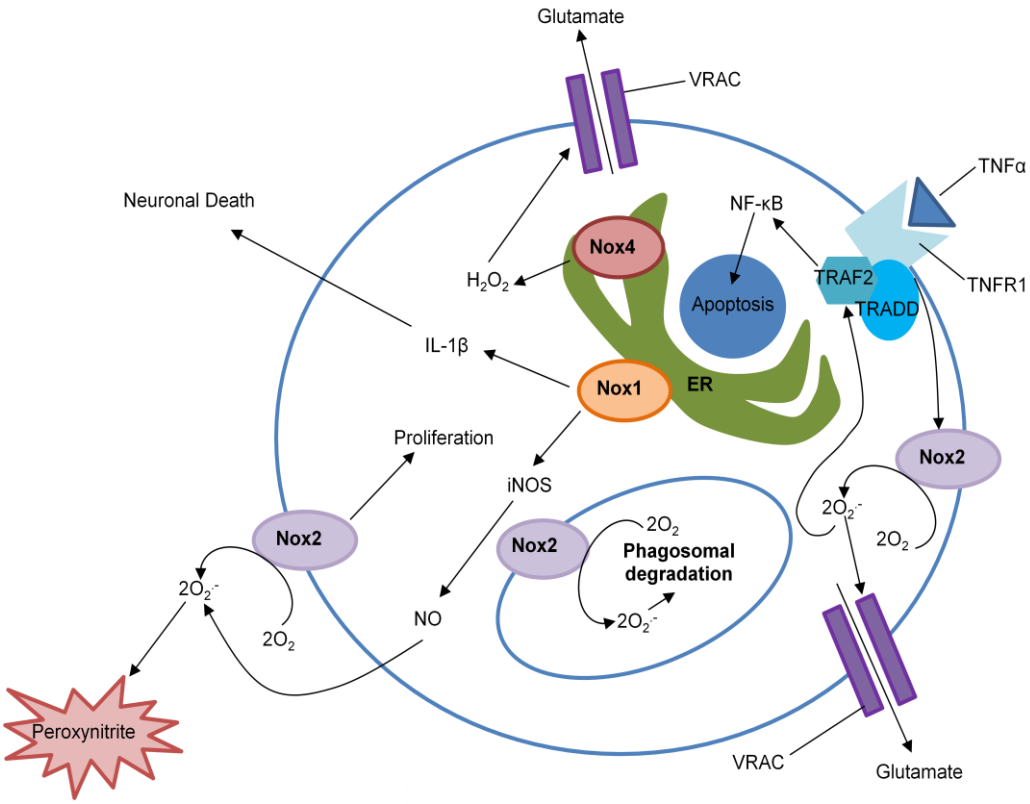

Figure 1.10 Known ramifications of microglial Nox1, Nox2 and Nox4 activation. *This schematic summarises the activities of the microglial Nox1, Nox2 and Nox4. Nox1 is localized to the ER and superoxide released from this NADPH oxidase isoform acts as a signalling molecule to induce iNOS expression, and subsequent NO production, which can react with superoxide to produce peroxynitrite, a potent neurotoxin. Nox1 also induces the expression and release of IL-1β, which is neurotoxic. Nox4 has been shown to be localized to the ER in microglia, where H_2O_2 released from this isoform activated voltage sensitive anion channels (VRAC) to induce the release of glutamate. Nox2 is expressed on phagosome membranes, where superoxide is released into the phagosome to increase acidity to aid degradation of debris. In addition, Nox2 is involved in the oxidative burst, releasing superoxide in response to pathogens. Nox2 is also implicated in proliferation, and the release of neurotransmitters such as glutamate. Nox2 also has an apoptotic role, whereby TNFα has been shown to induce superoxide production from Nox2, which then promotes the association of TRAF2 to the TNFR1/TRADD complex, to promote NF-κB mobilization to the nucleus where it induces cell death.*

1.5.4.1.2 Microglial NADPH oxidase in pathology

Superoxide production following activation of the microglial NADPH oxidase is implicated in regulating the expression of pro-inflammatory factors, whilst the release of superoxide is implicated in the oxidative burst and the removal of invading pathogens. NADPH oxidase activation can represent a protective mechanism, however, excessive or aberrant superoxide signaling can be detrimental to the survival of microglia themselves, and surrounding neurons and glia. Microglial intracellular superoxide production acts as a second messenger to regulate down-stream signaling pathways, such as PKC (Mander & Brown 2005), MAPK (Guyton et al. 1996) and NF-κB (Schreck et al. 1991). The activation of these signaling pathways modulates microglial reactivity, which promotes microglial mediated neurotoxicity. Furthermore, microglial superoxide release is a critical event in inflammation related neurotoxicity (Jeohn et al. 1998), which is supported by findings that toxins such as rotenone (Gao et al. 2002), paraquat (Wu et al. 2005) and Aβ (Qin et al. 2002) promote or enhance neurotoxicity through the generation of superoxide and microglial activation. Furthermore, SOD or catalase mimetics, which remove superoxide or H_2O_2 respectively, reduce LPS induced dopaminergic neurotoxicity, lending further evidence to the suggestion that superoxide and H_2O_2 play a central role in microglial mediated neurotoxicity (Wang et al. 2004).

The microglial expression and activity of Nox1 promotes a neurotoxic phenotype. Microglial Nox1 is activated by the yeast cell wall component zymosan and the bacterial endotoxin lipopolysaccharide (LPS) which promotes IL-1β and NO release in Nox2 knockout cells, suggesting that the production of these neurotoxins is a consequence of Nox1 activity (Chéret et al. 2008). Microglial Nox1 is also activated by angiotensin II (AngII), implicating Nox1 in vascular dementia (Dikalov et al. 2008). Furthermore, AngII signalling through Nox1

activity modulates CNS blood flow through mediating changes in blood preassure, which is detrimental to neuronal survival (Infanger et al. 2006).

Most research into microglial NADPH oxidase has focussed on the activity and expression of Nox2, which is implicated in many neurodegenerative conditions. Microglial Nox2 is up-regulated in AD (Block 2008), PD (Gao et al. 2003), ischaemia (Hur et al. 2010) and traumatic brain injury (TBI) (Dohi et al. 2010), and is also implicated in the clearance of viruses and bacteria (Ano et al. 2010; Clement et al. 2010). Studies have shown that LPS significantly elevates Nox2 induced superoxide production in microglia after 3 h exposure, which correlated with an increase in IL-6, IL-1β and TNFα production (Clement et al. 2010). Previous studies have shown that inhibition of LPS binding to toll-like receptors (TLR) prevents ROS production and the release of these inflammatory mediators (Liu et al. 2000). In addition to promoting superoxide production through Nox2 signalling, initiating intracellular signalling cascades through the activation of NF-κB and subsequent cytokine release (Zhang et al. 2010), LPS also stimulates iNOS expression and NO production, which synergises with microglial NADPH oxidase derived released superoxide to produce the potent neurotoxin peroxynitrite (Brown 2007), and is seen in AD transgenic mice in which NO production and Nox2 activation promotes neurotoxicity (Park et al. 2008). In addition to activation by bacterial infection, microglial Nox2 is activated in viral infections, such as encephalomyocarditis virus (EMCV), in which microglial production of superoxide causes bystander damage to neurons, leading to neuronal death (Ano et al. 2010).

Microglial Nox2 is heavily implicated in the neurotoxicity associated with neurodegenerative disease, and promotes the pathogenesis of inflammatory degenerative conditions such as AD and PD. Aβ can activate Nox2, promoting assembly of the functional enzyme, leading to an inflammatory phenotype associated with the production of superoxide radicals (Akiyama et al. 2000). This is supported by findings that superoxide production following exposure of

microglia to Aβ can be attenuated by inhibition of PI3-K (Bianca et al. 1999), shown to phosphorylate p40phox which is unique to Nox2 (Ellson et al. 2008). Nox2 activation in AD contributes to cytokine release resulting in neuronal apoptosis (Combs et al. 2001), which promotes AD progression. Nox2 activation by Aβ leads to the production of intracellular ROS, which acts as a signalling molecule to promote the expression and release of pro-inflammatory cytokines (Forman & Torres 2002), in particular TNFα, which can be abolished by the treatment of microglia exposed to Aβ with the NADPH oxidase inhibitor apocynin (Jekabsone et al. 2006). Furthermore, the release of cytokines such as TNFα from Aβ activated microglia can act on TNFα receptors on microglia in an autocrine manner, promoting further activation of Nox2, and increased microglial reactivity in AD (Mander et al. 2006). Further support for the suggestion that microglial Nox2 is implicated in AD comes from studies in which treatment of primary microglia with ibuprofen inhibits Aβ stimulated tyrosine phosphorylation which promotes Nox2 assembly, thereby attenuating superoxide production and microglial mediated neurotoxicity (Wilkinson et al. 2010). These data are supported by epidemiological studies demonstrating that long-term non-steroidal anti-inflammatory (NSAI) therapy reduces the risk of AD (McGeer et al. 1996). Furthermore, studies have linked the inflammatory microglial response and Nox2 mediated superoxide production in AD with neuronal damage in mouse models of AD (Wilkinson et al. 2010), thereby suggesting that Nox2 is an important mediator of AD progression.

Activation of the microglial Nox2 is also associated with PD. Epidemiological studies have shown that idiopathic PD is associated with exposure to pesticides (Priyadarshi et al. 2000), and the herbicide rotenone induces dopaminergic neuronal degeneration (Betarbet et al. 2000). Furthermore, rotenone promotes microglial NADPH oxidase activation, which increases the oxidative death of neurons in the SN (Gao et al. 2002). Furthermore, rotenone induced microglial superoxide production and subsequent neuronal death could be inhibited

with the NADPH oxidase inhibitor apocynin, suggesting the involvement of microglial Nox2 in the death of dopaminergic neurons seen in idiopathic PD (Gao et al. 2002). The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is also implicated in the pathogenesis of PD, and is used as a model of the disease. MPTP elevates gp91phox expression in the SN of mouse models of PD, which correlates with increased oxidative damage and neuronal death (Wu et al. 2003). Furthermore, in gp91phox knockout mice, injection of MPTP did not induce as severe Parkinsonism symptoms as wild type mice, (Wu et al. 2003), and blockade of the microglial NADPH oxidase with minocycline also conferred neuroprotection in the MPTP model of PD through inhibiting the release of IL-1β (Wu et al. 2002), which is a characteristic of Nox1 activation (Chéret et al. 2008), suggesting a role for Nox1 and Nox2 in PD.

In vitro studies have shown that treatment of microglia with MPTP enhanced reactive microgliosis in an NADPH oxidase dependent manner, as co-treatment with MPTP and the NADPH oxidase inhibitor apocynin significantly reduced microglial reactivity (Gao et al. 2003). Moreover, this also protected dopaminergic neurons in co-culture from microglial mediated death, suggesting an important role for the microglial NADPH oxidase in dopaminergic neuronal death in PD (Gao et al. 2003). The herbicide paraquat, which has structural similarity to MPTP, can also induce idiopathic PD (Peng et al. 2005) through NADPH oxidase activation and superoxide production in microglia (Bonneh-Barkay et al. 2005). Studies using BV2 microglia (a murine microglial cell line) have shown that paraquat induces microglial extracellular signal regulated kinase (ERK1/2) signalling, which, as part of the MAPK pathway, mediates phosphorylation of the Nox2 cytoplasmic subunits and promotes assembly of the functional enzyme and subsequent microglial superoxide production and neuronal death (Miller et al. 2007). In support of this, the growth factor TGFβ attenuates microglial production of superoxide and Nox2 assembly by inhibition of ERK1/2

in microglia exposed to MPTP (Qian et al. 2008). These studies therefore implicate the microlglial Nox2 in the progression of idiopathic PD.

Microglial Nox2 is also implicated in ischaemia and TBI. Nox2 inhibition is protective in *in vivo* models of ischaemia reperfusion, in which treatment with apocynin or gp91phox knockout mice showed a significantly reduced infarction volume than wild type mice (Chen et al. 2009). Furthermore treatment with the NADPH oxidase inhibitor apocynin improved the outcome of transient middle cerebral artery occlusion in a mouse model of stroke, supporting the importance of NADPH oxidase in ischaemia (Tang et al. 2008). In addition, chemical ischaemia promotes microglial NADPH oxidase activation *in vitro,* promoting the release of neurotoxic factors in an NADPH oxidase dependent manner, as siRNA knock-down of gp91phox in microglia attenuated this microglial mediated neurotoxicity (Hur et al. 2010). Mechanistically, it has been determined *in vitro* that the increase in superoxide release from activation of the microglial NADPH oxidase during re-oxygenation after ischaemia is due to the influx of calcium ions through voltage gated channels (Spranger et al. 1998), suggesting that microglial production of superoxide may contribute to reperfusion injury. In addition to the involvement of Nox2 in ischaemia, microglial reactivity and Nox2 expression and activation is up-regulated in TBI, in which gp91phox knock-out mice are protected against neuronal damage following injury (Dohi et al. 2010). These data therefore suggest an involvement of Nox2 in ischaemia and TBI, and suggest that pharmacological inhibition of Nox2 may be protective in these circumstances.

Nox4 has also more recently been identified in microglia (Harrigan et al. 2008), and there is increasing data linking its activity and expression to glioma, ischaemia, and the release of inflammatory cytokines following microglial activation *in vitro.* Microglial Nox4 expression is up-regulated following activation with zymosan, which induced superoxide production that could only be inhibited by the Nox4 inhibitor thioridazine (Harrigan et al. 2008).

Furthermore, microglial Nox4 activation mediates glutamate release through VRACs (Harrigan et al. 2008), which is implicated in chronic glutamate toxicity to cortical neurons, and is associated with several neurodegenerative conditions (Ha et al. 2010). In addition, *in vitro* studies using human microglia have shown that Nox4 activity is associated with constitutive generation of the pro-inflammatory cytokine IL-6, which contributes to neurotoxicity (Li et al. 2009). These studies have suggested that under certain pathological circumstances, the source of NADPH oxidase derived superoxide could switch from the inducible Nox2 isoform to the constitutive Nox4 isoform, which correlates with constitutive expression of pro-inflammatory cytokines and increased neurotoxicity, thereby suggesting that NADPH oxidase expression may contribute to the neurotoxic or neuroprotective phenotype of microglia (Li et al. 2009). This switch between NADPH oxidase isoform expression in microglia has also been demonstrated in models of stroke in rats. Nox4 is expressed initially at 6 h post-ischaemia, whilst Nox2 expression is up-regulated from 24 h (McCann et al. 2008). Furthermore, most damage following stroke occurs immediately after injury, therefore supporting the suggestion of a neurotoxic role for microglial Nox4 expression, and initial Nox4 mediated microglial neurotoxicity followed by a Nox2 mediated neuroprotective phenotype (McCann et al. 200). Nox4 expression is up-regulated in gliomas, and is implicated in cell proliferation, where Nox4 activation induces pro-survival signalling cascades such as Akt (Shono et al. 2008). Research into the involvement of microglial Nox4 in neurodegeneration is relatively recent, however, the findings outlined here suggest a role for Nox4 in mediating signalling involved in survival and the release of cytokines suggests that Nox4 may be an important regulator of microglial mediated neurotoxicity or survival.

1.6 Neurotransmitter and neurotransmitter receptor dysregulation in neurodegeneration

The regulation of neurotransmitter release by activation of neuronal presynaptic receptors is fundamental to neuronal communication (Patel et al. 2001). In addition to their role in mediating fast neuronal signalling, neurotransmitters themselves can also act as neurotoxic or neuroprotective substances (Kuhn et al. 2004), and the dysregulation of neurotransmitter release is implicated in several neurodegenerative conditions (Greenfield & Vaux 2002), affecting both neurons and the reactivity of surrounding microglia. Glutamate can act as a neurotoxic substance, causing neuronal death through excitotoxicity in ischaemia (Choi & Rothman 1990), whilst gamma-aminobutyric acid (GABA) is neuroprotective, and in a model of white matter anoxia , GABA attenuates neuronal dysfunction through $GABA_B$ receptors (Fern et al. 1995). Neurotransmitters released during neurodegenerative diseases can act on microglia, which express a wide repertoire of neurotransmitter receptors (Pocock & Kettenmann 2007). Neuronal stress seen in neurodegeneration often leads to metabolic dysfunction and release of ATP, which activates microglia, promoting chemotaxis to the site of neuronal injury (Färber & Kettenmann 2005), in addition to inducing NADPH oxidase activation (Parvathenani et al. 2003). Glutamate release from astrocytes and neurons is aberrant in AD and also in acquired immune deficiency syndrome (AIDS) dementia (Rossi & Volterra 2009), and glutamate release from astrocytes acts on microglia to induce TNFα release, which has neurotoxic consequences (Bezzi et al. 2001) . GABA is also dysregulated during injury, and elevated levels of GABA have been observed in patients with ischaemia, TBI or subarachnoid haemorrhage (Hutchinson et al. 2002). Furthermore, GABA can act on microglia to modulate the release of interleukins (Kuhn et al. 2004). GABAergic signalling is also dysregulated in AD, and excess GABA in the extracellular space is taken up by microglia leading to their activation (Casamenti et al. 1999).

1.6.1 Glutamate

The excitatory action of glutamate was first recognised by Hayashi (1952), and in the 1970's, glutamate was established as the main excitatory neurotransmitter in the CNS. Glutamate is implicated in development and learning and memory, however, dysregulation of glutamate signalling is seen in neurodegeneration, and its excessive release is a hallmark of acute excitotoxic injury seen in stroke and ischaemia, as well as in chronic conditions such as AD. Glutamate is released from neuronal pre-synaptic terminals in vesicles in a Ca^{2+} dependant manner, involving N and P/Q type voltage dependant Ca^{2+} channels which are closely linked to vesicle docking sites (Birnbaumer et al. 1994). Released glutamate is taken up by transporters, or binds to receptors which are expressed on both neurons and glia. Glutamate transporters are Na^{+} dependant, where one molecule of glutamate is co-transported with three Na^{+} and one H^{+} with the counter-transport of one K^{+} (Levy et al. 1998). Five glutamate transporters have been identified in the mammalian CNS, two of which are expressed in glia (the glutamate aspartate transporter – EAAT1, and glutamate transporter 1- EAAT2) and three in neurons (excitatory amino acid transporter - EAAT1, EAAT4 and EAAT5), which are linked to a Cl^{-} channel which opens when glutamate binds leading to membrane hyperpolarisation (Meldrum 2000). There are two classes of glutamate receptor, also expressed on both neurons and glia, namely the ionotropic glutamate receptors (iGluRs) and the metabotropic glutamate receptors (mGluRs), which will be discussed in greater detail later. Briefly, iGluRs are ion channels, which are opened upon ligand binding and depolarisation of the plasma membrane, which facilitates the influx of ions such as K^{+}, Na^{+} and Ca^{2+} to promote an action potential, or mediate down-stream effects through Ca^{2+} signalling. mGluRs are G-protein coupled receptors (GPCRs), which activate intracellular second messengers such as PLC, or adenylate cyclase upon ligand binding, which have a diverse range of effects on the cell (Meldrum 2000).

Glutamate dysregulation is central to several neurodegenerative disorders and excess glutamate release from neurons contributes to acute neurodegenerative conditions such as ischaemia and TBI, as well as influencing cell death in chronic conditions such as AD and PD. In acute conditions such as stroke, glutamate acting on neuronal *N*-Methyl-D-aspartic acid (NMDA) and 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid (AMPA) receptors as well as mGluR1 induces cell death, due to ionic disequilibrium related to excessive entry of Na^{+} and Ca^{2+} through ligand gated voltage channels. Calcium influx promotes the activation of apoptotic signalling pathways, such as those inducing iNOS expression, PLC activation and caspase cleavage, inflammation and necrotic cell death (Meldrum & Garthwaite 1990). Chronic and late-onset neurodegenerative conditions may also be partially due to glutamate dysregulation. In amyotrophic lateral sclerosis (ALS) patients, a reduction in the expression of GLT1 has been detected in the spinal cord and in brain regions showing a loss of motorneurons (Rothstein et al. 1995). Furthermore, a contributing factor to Huntington's disease (HD) pathology may be metabolic injury that causes striatal neurons to become vulnerable to excitotoxicity through NMDA receptor activation, and excessive activation of glutamate receptors in HD also leads to increased glutamate release and reduced uptake by glia, resulting in neuronal death (DiFiglia 1990).

There have been many studies demonstrating impaired glutamate uptake by glia in disease. Fibrillar Aβ (fAβ) impairs glutamate transport, which has been linked to lipid peroxidation and release of TNFα from microglia (Mattson & Chan 2003), which contributes to neuronal death. Furthermore, glutamate concentrations in the CSF of MS patients are increased in comparison to controls, which is also associated with the severity and course of the disease (Stover et al. 1997). Elevated glutamate CSF levels are also a hallmark of AD, and Aβ induces microglial activation which mediates glutamate release through the reversal of glutamate transporter function, which is potentiated under pathophysiological conditions

(Noda et al. 1999). Furthermore, microglia produce ROS under pathological conditions, which has also been linked with an increase in glutamate concentration in the extracellular space, as free radicals inhibit glutamate transporter efficacy, leading to a reduction in the uptake and clearance of extracellular glutamate in pathological conditions (Volterra et al. 1994). In addition, activation of the microglial Nox4 in pathology induces glutamate release which could contribute to disease pathology (Harrigan et al. 2008).

The regulation of glutamate signalling has great importance in the correct functioning of the CNS, and as shown here, dysregulation of this system can have damaging effects. The role of glia in the uptake and release of glutamate is also critical for the survival of neurons, and when this is aberrant, the release of glutamate or excessive activation of microglia by glutamate can have serious neurotoxic consequences.

1.6.2 GABA

GABA is an inhibitory neurotransmitter in the CNS which acts at inhibitory synapses and mediates membrane hyperpolarisation or depolarisation to regulate neuronal excitability (Roach et al. 2008). GABA is responsible for the migration of neuronal and glial precursor cells from the neural tube in the developing brain (Marczynski 1998), and also mediates the proliferation of neural progenitor cells, and the migration, differentiation and elongation of neurites (Sang et al. 2002). GABA is also implicated in the growth of embryonic and neural stem cells, through mediating BDNF expression (Wang et al. 2008).

Dysregulation of the GABAergic system is implicated in several neurodegenerative diseases, such as AD, HD and also AIDS dementia, in addition to promoting normal ageing (Greenfield and Vaux 2002). GABAergic and glutamatergic signalling are finely balanced in the normal brain, however, during ageing and in patients with AD, neuronal degeneration causes a reduction in ATP, which in turn increases the production of glutamic acid

decarboxylase (GAD), the enzyme responsible for GABA synthesis from glutamic acid, leading to an increase in GABA production in these conditions (Bao et al. 1995). Post-mortem analyses of AD brains have shown increases in GAD activity of up to 105% (Reinikainen et al. 1988), suggesting significant dysregulation of glutamatergic and GABAergic signalling. Furthermore, excess GABA is exocytosed from neurons in AD pathology, which can act on presynaptic $GABA_A$ receptors exacerbating neuronal death (Marczynski 1998). The increase in GABA tone seen in AD lowers brain metabolic function, resulting in further neuronal loss and neurodegeneration (Marczynski 1998). Furthermore, *in vitro* studies have shown that chronic treatment of neurons with GABA, mimicking the effect of the increased GABA tone in AD, increased neuronal vulnerability to glutamate toxicity (Erdö et al. 1991). Increased GABA activity is therefore detrimental in chronic neurodegenerartive conditions.

There are few reports regarding the effect of GABA on microglia and subsequent ramifications for neuroinflammation or neurodegeneration. However, microglia express functional $GABA_B$ receptors (Kuhn et al. 2004), and also respond to the $GABA_A$ receptor agonist muscimol (Cheung et al. 2009). GABA has been reported to be neuroprotective by attenuating neuronal dysfunction, and may also be protective through interactions with the microglial $GABA_B$ receptor (Kuhn et al. 2004).

1.6.3 ATP

ATP is a key metabolite in all cell types. It is a multifunctional nucleotide used as a coenzyme, and is required for energy transfer in the mitochondrial electron transfer chain. ATP is also a substrate in signal transduction pathways and is converted into the second messenger cyclic AMP (cAMP) by adenylate cyclase. In the CNS, ATP is an excitatory neurotransmitter, which was first described in 1959, when it was shown that ATP was released from nerves upon stimulation (Holton 1959). Furthermore, Holton(1959) showed

that ATP could be co-released with noradrenaline from sympathetic nerve endings, and Burnstock (1972) confirmed that ATP is synthesised and stored in presynaptic terminals, is released upon nerve stimulation, can be rapidly degraded by coenzymes, and pharmacological agents used to inhibit the effects of endogenous ATP can also suppress nerve stimulation. The excitatory action of ATP was determined by (White 1978), who showed ATP release from synaptosomes could be induced by high extracellular potassium concentrations, and also that depolarisation of synaptosomes lead to ATP release. Addition of ATP to cultured dorsal horn neurons also induced depolarisation, further confirming the excitatory action of ATP (White, 1978).

ATP mediates neuronal-glial interactions, and is released from both neurons and glia (North & Verkhratsky 2006). Glia and neurons express both metabotropic (P2Y) and ionotropic (P2X) purinergic receptors, which elicit a variety of effects. The ability of neuronal derived ATP to act on glial cells enables glia to respond to neuronal activity. Astrocytes and oligodendrocytes respond to ATP through the metabotropic P2Y purinergic receptors, which facilitates the release of intracellular calcium and glial reactivity (Verkhratsky et al. 1998). Microglia express both P2Y and P2X receptors, and predominantly respond to ATP released from damaged neurons or glia (Möller et al. 2000).

ATP has a wide ranging action on neurons and glia, and modulates neurite outgrowth, glial cell proliferation, microglial activation and induction of glial scar formation (Neary 1996). Under non-pathological conditions, ATP is present in the CNS at the synaptic cleft at concentrations of 100 μM (Silinsky 1975), however levels increase during neuronal damage, ischaemia and hypoxia (Rathbone et al. 1999), and microglia respond to ATP levels of 1 mM, which acts at the ionotropic P2X7 receptor with neurotoxic consequences (Parvathenani et al. 2003). Local increases in ATP concentrations at sites of neuronal injury induce microglial reactivity through the activation of a cation conductance mediated by P2X receptors, and with

a delayed activation of an outward directed potassium current mediated by P2Y receptors (Walz et al. 1993).

In pathological conditions, neuronal release of ATP into the microenvironment induces microglial activation and pathology, such as the production and release of superoxide (Parvathenani et al. 2003). Application of 1 mM – 5 mM ATP to microglia induces TNFα release in a calcium dependant manner triggered by ERK and JNK signalling (Hide et al. 2000), and also induces IL-1β release (Takenouchi et al. 2009), which is neurotoxic (Färber & Kettenmann 2006). Exposure of microglia to high levels of ATP is implicated in the progression of neurodegeneration and neuroinflammation, and increased levels of ATP induce the release of microglial response factor 1 (MRF-1), which is a 177 amino acid peptide up-regulated in response to neuronal death, which mediates a variety of microglial actions (Tanaka & Koike 2002); furthermore, microglial production of the endocannabinoid 2-arachidonylglycerol (2-AG) is increased upon exposure to ATP, which mediates neuroinflammation (Färber & Kettenmann 2006). In addition, prolonged exposure of microglia to ATP results in microglial necrosis, whilst acute exposure favours microglial proliferation (Takenouchi et al. 2009). It should however be stressed that only high levels of ATP induce microglial activation and a neurotoxic phenotype. Microglia stimulated with low levels of ATP, in the range of 30-50 μM release neuroprotective moieties such as plasminogen and other neurotrophic factors (Inoue 2002).

Physiological concentrations of ATP mediates microglial proliferation and chemotaxis and is neuroprotective (Honda et al. 2001). Furthermore, ATP regulates the movement of microglial fine processes in the resting state, as shown by injection of ATP into the thinned skulls of mice and the imaging of GFP tagged microglia, which exhibited a rapid chemotactic response (Davalos et al. 2005). Microglia can sense astrocyte activity through purinergic receptors, and calcium waves propagated in astrocytes can induce potassium currents in microglia, which

mimicked the response to P2Y receptor activation (Schipke et al. 2002). In this way, microglia use ATP to sense changes in their microenvironment, and can respond to pathological changes in ATP levels seen in areas of neuronal death, and also to physiological concentrations to promote neuroprotection and the release of neurotrophic factors.

1.6.4 Neurotransmitter receptor modulation of microglial activation in neurodegeneration and involvement of ROS

Microglia express a range of neurotransmitter receptors, and respond to a variety of neurotransmitters, inducing the release of neuroprotective or neurotoxic factors (Pocock & Kettenmann 2007). As microglia lack synaptic structures, neurotransmitters modulate microglial reactivity through a model of 'volume transmission' (Agnati et al. 1995), in which neuro-active substances diffuse throughout the extracellular space to activate extrasynaptic receptors (Pocock & Kettenmann 2007). Neurons express neurotransmitter receptors at extrasynaptic regions, and the action of neurotransmitters on these extrasynaptic receptors has important roles in the regulation of the neural network, however, neurotransmitters released into the extracellular space may also activate microglial neurotransmitter receptors, with consequences for microglial reactivity (Pocock & Kettenmann 2007).

Modulation of neurotransmitter receptors on microglia has a wide range of effects on microglial reactivity (Table 1), and cytokine, chemokine and ROS production. Microglia express glutamate, GABA and purinergic receptors of both the ionotropic and metabotropic subclasses, in addition to adenosine, cholinergic, cannabinoid, adrenergic, dopamine and opioid receptors, as well as neuropeptide receptors (reviewed in Pocock & Kettenmann 2007). The main focus of this thesis however is on glutamatergic, GABAergic and purinergic receptor modulation on microglial activation and superoxide production, which will be described in more detail.

The modulation of microglial metabotropic glutamate receptors has a diverse range of effects on microglial reactivity and the surrounding environment. Microglia express the group I mGluR1 and mGluR5a receptors (Table 1), which couple to G_q proteins, and promote PLC-1β activation, resulting in DAG and Ins-1,4,5-P_3 production, followed by PKC activation and the opening of IP_3 gated calcium channels on the ER, leading to a rise in intracellular calcium, which has a wide range of downstream effects (Ribeiro et al. 2010).

There are scant data regarding the role of mGluR group I modulation on microglial activation, however treatment of microglia with the mGluR5 specific agonist *trans*-(1*S*,3*R*)-1-Amino-1,3-dicarboxycyclopentane (ACPD) elevates intracellular calcium concentrations (Biber et al. 1999). Microglial group I mGluR expression is modulated in neuropathology. Microglial mGluR5 expression is down-regulated in the MS brain (Geurts et al. 2003), which may contribute to disease progression. Furthermore, microglial mGluR5 activation attenuates NADPH oxidase activity and expression of the p22phox and p47phox subunits, and also reduces LPS-generated ROS production (Loane et al. 2009), in addition to inhibiting the release of LPS-stimulated pro-inflammatory mediators such as TNFα and NO, thereby suggesting that activation of mGluR5 may down-regulate microglia with neuroprotective consequences (Farso et al. 2009). This mGluR5 mediated inhibition of NADPH oxidase activity also attenuated MAPK, NF-κB and STAT1 signalling, thereby down-regulating the expression and release of inflammatory modulators (Loane et al. 2009). Furthermore, *in vivo* models of rat spinal cord injury demonstrate that treatment with the mGluR5 agonist **(***RS*)-2-Chloro-5-hydroxyphenylglycine (CHPG) enhanced recovery through down-regulation of NADPH oxidase activity when compared with untreated controls (Byrnes et al. 2009). These data therefore suggest that activation of the microglial group I mGluRs could promote neuroprotection through reducing microglial reactivity and superoxide production.

Microglia also express the group II and III mGluRs, which are negatively coupled to adenylate cyclase (Table 1). Of the group II receptors, microglia express mGluR2 and mGluR3 (Taylor et al. 2002; Taylor et al. 2005), and mGluR3 activation is protective and down-regulates microglial activation (Taylor et al. 2005). However, microglial mGluR2 activation is neurotoxic, mediating microglial stress characterised by mitochondrial depolarisation and TNFα and Fas ligand release, which induce neuronal caspase 3 activation and subsequent apoptosis through binding to TNF receptor 1 (TNFR1) (Taylor et al. 2005). In addition, activation of microglia with CgA and Aβ induce glutamate release from microglia (Kingham et al. 1999), which feeds back onto microglial group II mGluRs to exacerbate the release of neurotoxic factors (Taylor et al. 2002). Furthermore, activation of microglial group II mGluRs enhances CgA mediated neuronal death, which could be attenuated by group II mGluR inhibition, suggesting that modulation of this receptor promotes the production and release of neurotoxic substances in AD (Taylor et al. 2002). The microglial reactivity observed during AD promotes glutamate release at concentrations that can feedback onto microglial group II mGluRs, which exacerbates microglial mediated neurotoxicity.

Modulation of the microglial group II mGluRs is implicated in MS, and myelin induced microglial neurotoxicity can be exacerbated by modulation of group II mGluRs. Stimulation of microglia with (2*S*,2'*R*,3'*R*)-2-(2',3'-Dicarboxycyclopropyl)glycine (DCG-IV) (a group II agonist) is neurotoxic, however neurotoxicity following exposure of microglia to myelin was also enhanced by co-exposure of microglia to the group II mGluR agonist (Pinteaux-Jones et al. 2008). Furthermore, this neurotoxicity could be reduced upon treatment of microglia with myelin and the group II mGluR antagonist (*RS*)-α-Methyl-4-carboxyphenylglycine (MCPG) suggesting a role for the group II mGluRs in MS disease progression (Pinteaux-Jones et al. 2008). There are however differing reports regarding the role of the group II mGluRs in *in vivo* models of PD. Injection of DCG-IV into the striatum of MPTP treated rats elevates

BDNF expression in reactive microglia close to the injection site, which protected dopaminergic terminals against neurotoxicity (Venero et al. 2002). These findings suggest a protective role for group II mGluR activation, however the concentrations used and the *in vivo* nature of this model may account for the differences between this and the *in vitro* findings with this agonist.

Microglia express mGluR4, mGluR6 and mGluR8 of the group III subclass of mGluRs (Table 1), which respond to a higher level of glutamate than the group II receptors (Pin & Duvoisin 1995). Activation of these receptors is also negatively coupled to adenylate cyclase, is neuroprotective, and can attenuate the neurotoxicity seen following treatment of microglia with the mGluR group II agonists (Taylor et al. 2003). *In vivo* studies of MS lesions have shown that mGluR8 is up-regulated in microglia surrounding active, de-myelinating lesions, suggesting that microglial group III mGluRs may be implicated in MS progression (Geurts et al. 2005). The nature of these studies does not indicate whether mGluR8 up-regulation is protective or toxic, however, taken together with *in vitro* evidence, it could be suggested that mGluR8 expression and activity may be protective, and could represent an attempt to limit microglial toxicity and protect oligodendrocytes from glutamate toxicity (Geurts et al. 2005). In support of this suggestion, activation of the microglial group III mGluRs promotes the release of neurotrophic factors in a PKC dependent manner, which is neuroprotective (Liang et al. 2010).

Microglia also express subunits of the ionotropic glutamate receptor subclass, including subunits of the AMPA and NMDA receptors (Table 1). The predominant functional iGluR expressed in microglia is the AMPA receptor, and microglia express the GluR2 – GluR4 subunits (Noda et al. 2000). Little is known about the role of microglial AMPA receptors in neurodegenerative disease, however GluR4 subunits are up-regulated in microglia in the CA1 region of the hippocampus after transient forebrain ischaemia in the rat, although the

ramifications of this are yet to be determined (Gottlieb & Matute 1997). Microglial AMPA receptor activation mediates TNFα release, which could be attenuated by inhibition of the AMPA receptor with the antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Noda, 2000). As microglial TNFα release can be neurotoxic, it could be suggested that activation of the microglial AMPA receptors may promote neuronal death, and it has been shown that microglial AMPA induced TNFα release is toxic to oligodendrocytes (Merrill & Benveniste 1996).

Microglia also express NMDA receptor subunits, and resting and hypoxic rat microglial cells *in vitro* express the NR1, NR2A, NR2B, NR2C, NR2D and NR3A subunits (Murugan et al. 2011). Furthermore, NMDA receptors expressed on activated microglia are functional, as demonstrated by an increase in intracellular calcium upon treatment with glutamate and glycine, which could be attenuated by addition of the open channel blocker (5*R*,10*S*)-(-)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cylcohepten-5,10-imine-maleate (MK-801) (Murugan et al. 2011). The microglial NMDA receptor is also up-regulated after ischaemia, which promotes the translocation of NF-κB to the nucleus, resulting TNFα, IL-1β and iNOS expression (Murugan et al. 2011). Furthermore, *in vivo* studies have shown that the NR1 subunit of the NMDA receptor is up-regulated following ischaemic stroke in rats. Treatment of microglia with NMDA also increases the expression of neurotrophic factors such as BDNF, glial derived neurotrophic factor (GDNF) and NGF, in a PKC dependant manner (Noda et al. 1999; Liang et al. 2010). Production of these neurotrophic factors also promotes microglial glutamate release, which can feedback onto these cells and activate other glutamate receptors, or promote excitotoxicity (Matute et al. 2006), however this has not yet been investigated in great detail. The ramifications of microglial NMDA receptor activation therefore requires further investigation.

Microglia respond to GABA, and express the G-protein coupled $GABA_B$ receptors (Charles et al. 2003; Kuhn et al. 2004), however, they are also responsive to treatment with the ligand gated $GABA_A$ agonist muscimol (Synowitz et al. 2001) (Table 1). Immunocytochemisty revealed that activated microglia (detected by ED1 immunoreactivity) expressed $GABA_{B1a,}$ $GABA_{B1b}$ and $GABA_{B2}$ subunits, which sense extracellular GABA to potentiate inhibitory signals (Charles et al. 2003). However, immunocytochemisty showed that a high proportion of GABA immunoreactivity in microglia was concomitant with intracellular compartments and the nucleus, suggesting a role for GABA in the control of transcription, as the C-terminal tail of $GABA_B$ receptors interacts with the nuclear transcription factors ATF-4 (Vernon et al. 2001) and CREB-2 (White et al. 2000). Activation of microglial $GABA_B$ receptors may therefore activate transcription factors responsible for the induction of microglial reactivity (Charles et al. 2003). This suggestion is supported by findings that activation of the $GABA_B$ receptor on microglia modulates interleukin release (Kuhn et al. 2004). *In vivo* studies of the axotomized facial nucleus have shown elevated $GABA_B$ receptor expression in reactive microglia, and that these receptors are active, as treatment with the agonist 3-Aminopropyl(methyl)phosphinic acid (SKF-97541) transiently elevates intracellular calcium levels, that could be reversed by co-treatment with the antagonist baclofen (Kuhn et al. 2004). However, in contrast to the studies of Charles et al. (2003), $GABA_B$ receptors have also been found at microglial lamellaepodia (Kuhn et al. 2004), and the resulting activation of microglial $GABA_B$ receptors could attenuate LPS induced IL-6 and IL-12p40 release, thereby suggesting that microglial $GABA_B$ receptors modulate the inflammatory properties of microglia (Kuhn et al. 2004). This is in line with reports that GABA is neuroprotective, and *in vivo* models have demonstrated that GABA attenuates neuronal dysfunction through $GABA_B$ receptors (Fern et al. 1995). The findings that activated microglia express $GABA_B$ receptors and that these receptors may be localised to the nucleus and subsequent

transcription factors may suggest that activation of the microglial $GABA_B$ receptor correlates with a neuroprotective phenotype, modulating the inflammatory response by down-regulating the release of inflammatory cytokines and protecting against neuronal death.

Microglia are also sensitive to $GABA_A$ receptor agonists and antagonists, however the existence of functional receptors is yet to be determined, and the $GABA_A$ receptor may only be expressed during pathology (Synowitz et al. 2001). Cells from glioma lines express high levels of the $GABA_A$ receptor after injection into the rat brain, suggesting that microglial $GABA_A$ receptor expression may be modulated by the microenvironment (Synowitz et al. 2001). Furthermore, only glioma cells grown in direct contact with CGCs showed a GABA response, and microgial $GABA_A$ receptor activation induced a chloride current and a long lasting blockade of potassium current, suggesting that activation of the $GABA_A$ receptor may down-regulate the microglial response (Synowitz et al. 2001). Activation of the microglial $GABA_A$ receptor with muscimol enhances the release of macrophage inflammatory protein-1α (MIP-1α), a chemokine implicated in acute inflammation (Cheung et al. 2009), superoxide production and oxidative stress (Louboutin et al. 2010). Furthermore, modulation of $GABA_A$ receptors also induces ROS production through Nox4, and subsequent expression of matrix metalloproteinase 9 (MMP9) which mediates BBB dysfunction allowing the influx of macromolecules to the CNS, which is seen in ischaemia (Tyagi et al. 2009).

Microglia express purinergic receptors of both the metabotropic (P2Y) and ionotropic (P2X) subtype (Table 1) (Pocock & Kettenmann 2007). Activation of microglial purinergic receptors promotes migration and cytokine release (Davalos et al. 2005), and activation of P2Y receptors modulates membrane ruffling and chemotaxis (Honda et al. 2001), whilst activation of the P2X receptors results in TNFα release (Suzuki et al. 2004), and stimulation NADPH oxidase derived superoxide production (Parvathenani et al. 2003).

RT-PCR analysis has shown that BV2 and primary microglia express $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ (Brautigam et al. 2005; Crain et al. 2009). P2Y receptors respond to different purines; the $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors are preferentially activated by adenosine diphosphate (ADP), whilst $P2Y_6$ receptor is activated by uridine diphosphate (UDP) and $P2Y_{2/4}$ is activated by ATP and uridine triphosphate (UTP) (Seo et al. 2008). The $P2Y_{12}$ receptor is implicated in microglial migration through adenylate cyclase activation which mediates morphological changes in microglia enabling them to respond to and migrate to sites of neuronal injury (Haynes et al. 2006). It has, however, been shown that microglial activation by LPS down-regulates $P2Y_{12}$ receptor expression, whereas in the facial nerve axotomy model of microglial activation $P2Y_{12}$ receptor expression is up-regulated, therefore suggesting that the induction of microglial motility through the $P2Y_{12}$ receptor is specific to activation of microglia by nucleotides rather than toxic peptides (Haynes et al. 2006). This has been further supported by findings that in $P2Y_{12}$ deficient mice, microglial membrane ruffling did not occur upon application of ADP or ATP, and microglia did not respond to local tissue damage resulting from laser ablation in these models, suggesting that the $P2Y_{12}$ receptor is the primary receptor implicated in microglial chemotaxis (Haynes et al. 2006).

Microglial phagocytosis is mediated through $P2Y_6$ receptor activation with UDP, which promotes activation of the PLC signalling pathway and a mobilisation of intracellular calcium (Koizumi et al. 2007). In the injured brain, particularly following excitotoxicity, UDP and UTP are released into the extracellular space and are taken up by microglia through $P2Y_6$ receptors (Kim et al. 2011). It has been shown that kainic acid increases extracellular UTP concentration to over 20 times normal levels (Lazarowski & Harden 1999) which also elevates extracellular UDP, as UTP is converted to UDP by ectonucleoside diphosphokinase (Zimmermann 2000). UDP acting on microglial $P2Y_6$ receptors induces the expression of the chemokines CCL2 and CCL3, and UDP acting on astrocytic $P2Y_6$ receptors aids the

recruitment of blood monocytes and microglia to sites of injury (Kim et al. 2011). Little is known about the ramifications of microglial $P2Y_{2/4}$ receptor activation, however, these receptors could be involved in the release of intracellular calcium, as they are coupled to PLC-1β signalling, which induces the opening of IP_3 gated calcium channels on the ER (Wang et al. 2000).

Microglia express the $P2X_4$ and $P2X_7$ receptor subtypes (Pocock & Kettenmann 2007). The microglial $P2X_4$ receptor is implicated in chemotaxis and pain pathways, and its expression is up-regulated in microglia of the spinal cord after nerve injury (Inoue 2006). Furthermore, $P2X_4$ receptor up-regulation maintains allodynia in rats injected with ATP activated microglia, suggesting that these activated microglia release ATP which acts at $P2X_4$ receptors to promote activation of pain pathways (Inoue 2006). The $P2X_7$ receptor differs from the other P2X receptors, in that its transient activation induces the opening of a large pore, permeable to molecules of up to 900 Da (Skaper et al. 2010). Furthermore, it is also implicated in TNFα release (Suzuki et al. 2004), and in superoxide production through activation of the NADPH oxidase (Parvathenani et al. 2003). The $P2X_7$ receptor is activated by higher concentrations of ATP (1 mM), which are released from neurons following neuronal injury (Pocock & Kettenmann 2007). ATP is the only known activator of the $P2X_7$ receptor, and microglial $P2X_7$ receptor activation after LPS treatment induces the release of IL-1β through a MAPK signalling cascade (Skaper et al. 2010). Furthermore, microglial $P2X_7$ receptor activation is implicated in IL-6 production, as demonstrated with $P2X_7$ knockout mice which do not produce IL-6 in response to treatment with ATP (Sperlágh & Illes 2007).

Activation of the microglial $P2X_7$ receptor is both protective and damaging, and regulation of the production of protective molecules such as plasminogen, TNFα and the endocannabinoid 2-AG; or toxic molecules such as IL-1β and NO could follow a time and concentration

dependant pattern, with the production of protective molecules occurring at earlier time points (Inoue 2002a). The $P2X_7$ receptor is implicated in neurodegenerative disease, and studies using AD transgenic mice have shown that the $P2X_7$ receptor is up-regulated in these conditions, which correlated with an increase in superoxide production in these animals in an NADPH oxidase dependent manner (Parvathenani et al. 2003). Furthermore, in microglia pre-treated with Aβ followed by application of ATP, a significant increase in IL-1β, IL-1α and IL-18 was also observed, suggesting a link between $P2X_7$ receptor activation and AD (Sperlágh & Illes 2007).

Receptor	Activity	Expression
Group I mGluRs: mGluR1 and mGluR5a	G_q coupled. Promote PLC-1β activation and opening of IP_3 gated intracellular calcium stores. Modulation of microglial group I mGluRs affects superoxide production.	Expressed *in vivo* (Geurts et al. 2003; Ribeiro et al. 2010) and *in vitro* (Loane et al. 2009).
Group II mGluRs: mGluR2 and mGluR3	$G_{i/0}$ coupled. Negatively coupled to adenylate cyclase. Microglial mGluR3 activation is neuroprotective, whilst mGluR2 activation is toxic.	Expressed *in vivo* (Venero et al. 2002) and *in vitro* (Taylor et al. 2002; 2005).
Group III mGluRs: mGluR4, mGluR6, mGluR8	$G_{i/0}$ coupled. Negatively coupled to adenylate cyclase. Microglial Group III mGluR activation is neuroprotective.	Expressed *in vivo* (Geurts et al. 2005) and *in vitro* (Taylor et al. 2003).
AMPA receptor	Ionotropic glutamate receptor – mediates Ca^{2+}, Na^{+}, and K^{+} influx. Modulates TNFα release from microglia.	Expressed *in vivo* (Gottlieb & Matute 1997), and *in vitro* subunits (Noda et al. 2000).
NMDA receptor	Ionotropic glutamate receptor – mediates Ca^{2+} influx. Modulates PKC activation and the release of neuroprotective factors.	Expressed *in vivo* and *in vitro* (Murugan et al. 2011).
$GABA_A$ receptor	Ionotropic GABA receptor. Mediates a Cl^{-} conductance, inhibits neurotransmission. In Microglia, mediates the release of MIP-1α and enhances inflammation.	Expressed *in vitro* (Synowitz et al. 2001). No evidence of *in vivo* expression to date.
$GABA_B$ receptor	Metabotropic GABA receptor. In microglia stimulates the activation of a K^{+} conductance and attenuates the nflammatory response.	Expressed *in vivo* (Vernon et al. 2001), and *in vitro* (Charles et al. 2003).
Ionotropic P2X receptors: P2X7, P2X4	Ionotropic P2X receptors promote elevated K^{+} conductance and Ca^{2+} influx. In microglia, P2X4 activation regulates membrane ruffling and chemotaxis, whilst P2X7 receptor activation promotes superoxide production.	Expressed *in vivo* (Inoue 2006) and *in vitro* (Parvathenani et al. 2003).
Metabotropic P2Y receptors: P2Y1, P2Y2/4, P2Y2, P2Y12 and P2Y14	Metabotropic P2Y receptors elevate intracellular Ca^{2+} levels via a G_q and PLC-1β pathway. Activation of microglial P2Y receptors mediates the movement of fine processes and chemotaxis.	Expressed *in vivo* (Kim et al. 2011) and *in vitro* (Brautigam et al. 2005; Crain et al. 2009).

Table 1. Neurotransmitter receptor expression on microglia

Modulation of microglial neurotransmitter receptors therefore affects microglial reactivity. Microglial $P2X_7$ receptor and mGluR5 activation directly modulates the NADPH oxidase (Fig. 1.11), with activation of mGluR5 inhibiting microglial NADPH oxidase activation and promoting protection, and activation of $P2X_7$ receptors exacerbating disease progression through superoxide production. As there is strong evidence to suggest that modulation of microglial neurotransmitter receptors mediates microglial activation, it was important to investigate whether this affected NADPH oxidase activation, as little is known of the consequences of modulation of other neurotransmitter receptor subtypes on NADPH oxidase activation and superoxide production (Fig. 1.11). The suggestion that receptor modulation alters microglial phenotype and that microglia are sensitive to changes in neurotransmitter levels could suggest that modulation of microglial neurotransmitter receptors may mediate NADPH oxidase activity which could have important consequences for the progression of neurodegenerative diseases. An investigation into the effects of modulation of neurotransmitter receptors on the microglial NADPH oxidase and the ramifications for neuronal survival could therefore provide further information on microglia in pathology, and may provide targets for pharmacological intervention in neurodegenerative conditions.

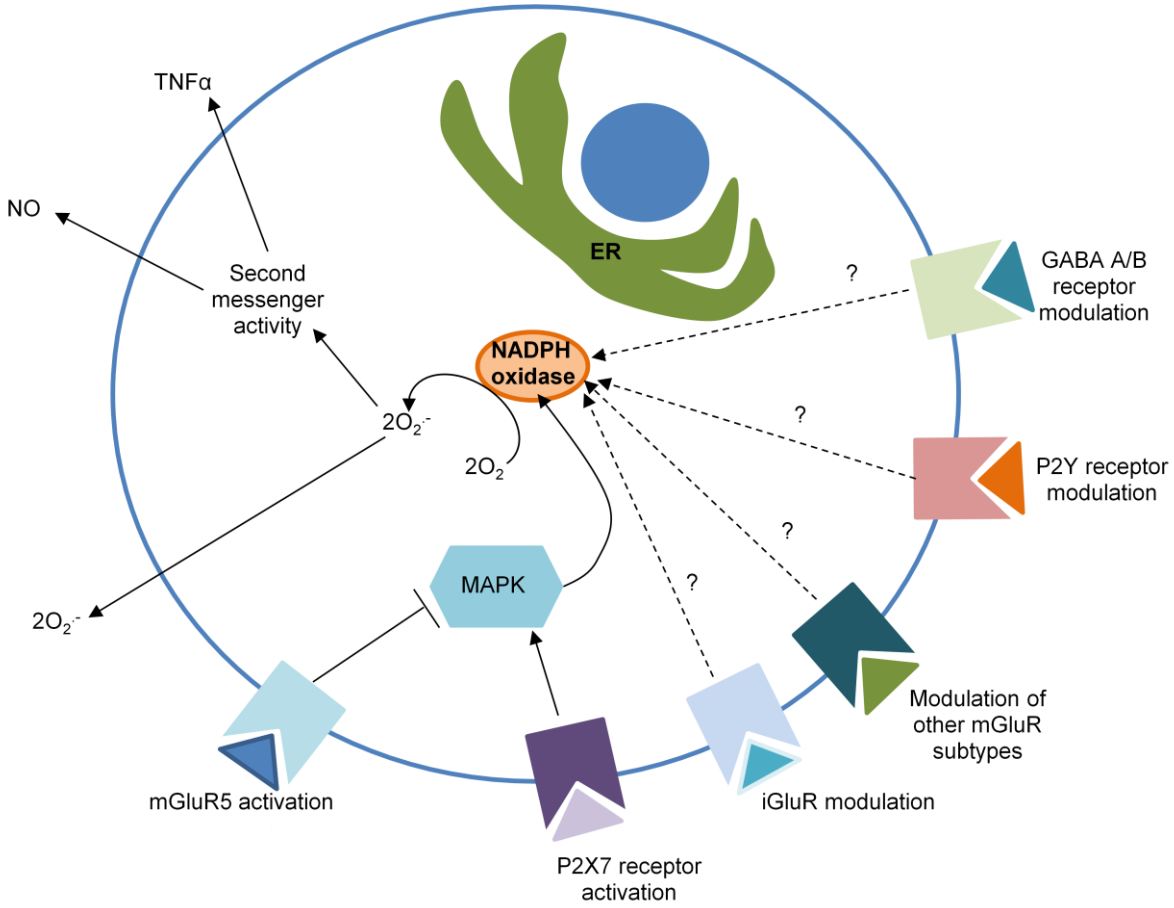

Figure 1.11 Summary of the activation of the microglial NADPH oxidase following modulation of neurotransmitter receptors. *To date, it has been shown that activation of the microglial mGluR5 down-regulates MAPK activity and prevents NADPH oxidase activity, thus reducing the release of TNFα and NO, which has neuroprotective consequences. Activation of the P2X7 receptor has been shown to up-regulate MAPK signalling and promote activation of the NADPH oxidase and release of superoxide, and is neurotoxic. It is not yet known whether modulation of other mGluR's, iGluR's. GABA or P2Y receptors modulates the NADPH oxidase, or the ramifications for neuronal survival.*

1.7 Aims and objectives

Microglial NADPH oxidase activation and subsequent superoxide production is implicated in the production and release of neurotoxic or neuroprotective factors in neurodegenerative conditions through the activation of different microglial signalling pathways. Furthermore, neurotransmitter dysregulation contributes to the progression of many neurodegenerative conditions, and microglia express a number of neurotransmitter receptors which mediate a variety of down-stream effects, including the modulation of the NADPH oxidase, which has ramifications for neuronal survival.

The aims of this thesis were therefore to investigate whether neurotransmitters or modulation of neurotransmitter receptors of the glutamatergic, GABAergic or purinergic subtypes could affect NADPH oxidase activation and subsequent superoxide production in BV2 and primary microglia. Superoxide production in BV2 and primary microglia was robustly measured using four assays: HPLC, flow cytometry, a plate based colorometric assay, and fluorescence microscopy, using two superoxide sensitive probes to validate findings and to ensure a comprehensive analysis of superoxide production. The signalling pathways involved in neurotransmitter and receptor mediated NADPH oxidase activation were also explored, with a focus on the p38MAPK and p44/42ERK signalling cascades, which are implicated in microglial activation and have been shown to induce NADPH oxidase activation. Furthermore, the NADPH oxidase isoforms expressed following modulation of neurotransmitter receptors were also investigated, as modulation of different Nox isoforms can mediate the release of protective or toxic factors. To investigate whether activation of the microglial NADPH oxidase, following modulation of neurotransmitter receptors, may promote the production and release of protective or toxic fators, microglial conditioned media assays were used to investigate the neuoprotection or toxicity on cerebellar granule neurons (CGCs). It was hoped that this research may provide some insight into whether microglial

NADPH oxidase derived ROS production, as a consequence of neurotransmitter receptor modulation, could mediate neuronal survival.

This research therefore aimed to provide an insight into the role of the microglial NADPH oxidase activation in pathology in which neurotransmitter signalling is unbalanced, and it was hoped that these findings could suggest potential targets for therapeutic intervention. Harnessing the ability of microglia to respond to modulation of neurotransmitter receptors in a protective or toxic manner through the production of ROS could represent an important target for pharmacological intervention in several severely debilitating neurodegenerative diseases.

Chapter 2

Materials and Methods

2.1 Materials

Sprage Dawley rat pups were obtained from University College London in-house colonies from Central Biological Services. The BV2 microglial cell line was a kind gift from Dr Claudie Hooper (Kings College London).

All chemicals and reagents were purchased from Sigma-Aldrich (Dorset, UK), unless otherwise stated. Foetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium (MEM), phenol-red free medium and accutase were obtained from Invitrogen (Paisley, UK). Tissue culture plasticware, filration units, syringes and glass Pasteur pipettes for tissue culture were obtained from Triple Red (Buckinghamshire, UK) and coverslips were obtained from Scientific Laboratory Supplies (Nottingham, UK). Glass microscope slides, Eppendorf tubes, 50 ml and 15 ml Falcon tubes, and serological pipettes were from VWR International (Leicestershire, UK). Phosphate buffered saline (PBS) powder was purchased from ICN Biomedicals (Maidenhead, UK).

Trizol, Tris-Borate-EDTA (TBE) buffer, Agarose, Superscript II Reverse transcriptase kit (containing superscript reverse transcriptase, oligo dT primer, RNase out, DTT, first strand buffer), and ethidium bromide were from Invitrogen (Paisley, UK). The PCR kit (containing Taq polymerase, magnesium chloride, and PCR buffer) was from Promega (Southampton, UK). PCR tubes and caps in strips were obtained from Applies Biosystems (Warrington, UK). Primers were designed using the Roche primer design tool, and were made by Sigma Custom Oligos (Dorset, UK).

Western blotting apparatus was from BioRad (Hertfordshire, UK). Acrylamide was from Severn Biotech (Worcestershire, UK). ECL reagent and PVDF membrane was from Millipore (Watford, UK). X-Ray film, developer and fixer solutions were from Kodak (supplier, Scientific Laboratory Supplies, Nottingham, UK). Flow cytometry reagents (FACS clean and sheath buffer) and consumables (FACS tubes) were from Becton Dickenson (Oxford, UK). HPLC vials were from VWR International (Leicestershire, UK).

Receptor agonists or antagonists and pathway inhibitors are listed in Table 1. Nitroblue tetrazolium chloride (NBT) was obtained from Calbiochem (Darmstadt, Germany), and dihydroethidium (dHEth), Hoechst 33243 nuclear stain, and Propidium Iodide (PI) were from Invitrogen (Paisley, UK). Vectashield was from Vector Labs (Burlingame, California). Antibodies are listed in Table 3 and Table 7.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 BV2 cell culture

The BV2 cell line was a kind gift from Dr Claudie Hooper (MRC Centre for Neurodegenerative Research, Institute of Psychiatry, Kings College London, UK) and was originally obtained from Dr FS Tzeng (Department of Life Sciences, National Cheng Kung University, Taiwan). BV2 cells are a mouse microglial cell line, immortalized by the inculsion of a v-raf/v-myc oncogene carrying retrovirus J2 (Blasi et al. 1990). The cells retain the morphological and phenotypical characteristics of primary microglial cells in culture conditions, and LPS treatment of BV2 microglia induces the up-regulation of inflammatory genes also seen following LPS treatment of primary microglia, suggesting that BV2 microglia respond in the same way as primary microglia to activating stimuli, however BV2 activation is less pronounced (Lund et al. 2006).

BV2 cells were maintained in culture medium (DMEM supplemented with 2 mM glutamine, 44 mM $NaHCO_3$, 10% FBS, and 100 U/ml penicillin, 100 μg/ml streptomycin) at 37ºC in a humidified atmosphere with 6% CO_2. BV2 cells were passaged twice weekly. Medium was removed and cells were washed with PBS made up in filtered, UV- treated dH_2O to remove excess medium. Cells were then detached from the culture flask using 2 ml 400 – 600 U/ml accutase and were incubated for 1 min at 37ºC to promote enzymatic activity. Accutase was neutralised using an equal volume of serum containing media, and cells were transferred to a 15 ml Falcon tube, then centrifuged at 3645 g for 5 minutes (Eppendorf Centrifuge 5804R). The pellet was resuspended in 5 ml medium, and cells were seeded into a new flask. BV2 cells were cryopreserved at $1x10^6$ cells / ml in medium composed of DMEM supplemented

with 2 mM glutamine, 44 mM $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, 20% FBS and 10% dimethyl sulphoxide (DMSO). Cells were snap frozen in dry ice, and transferred to a -80ºC freezer for long-term storage. Cells were thawed by defrosting one aliquot and transferring to a T25 culture flask in 10 ml pre-warmed media. Once cells had adhered, medium was changed to remove DMSO contamination, and cells were grown to confluency. Cells were passaged into larger T175 flasks before use.

BV2 cells were routinely counted using a haemocytometer and plated in 96 well plates at $1.0x10^4$ cells per well, in 24 well plates at $2.5x10^4$ cells per well and in 6 well plates at $1.0x10^5$ cells per well. Before treatments, the medium was changed to serum-free DMEM (DMEM supplemented with 2 mM glutamine, 44 mM $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin) for at least 3 h to down-regulate receptor expression, resulting in a ramified morphology similar to that seen in primary microglial cells in culture, which allowed cells to respond better to activating stimuli.

2.2.1.2 Primary microglial culture

Primary microglia were isolated from five day old Sprague Dawley rat pups using a method developed by Kingham et al. (1999). Eight post natal pups were killed by cervical dislocation and decapitation in accordance with the Scientific Proceedures Act 1986 (United Kingdom). Whole brains, minus the cerebellum which were used to culture CGCs (section 2.2.1.3), were removed and placed into 1 x PBS (137 mM NaCl, 5.37 mM KCl, 5.65 mM $NaH_2PO_4.H_2O$, 13.3 mM $Na_2HPO_4.7H_2O$, 11.1 mM D-glucose, 0.02% bovine serum albumin (BSA), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 3 μg/ml ampicillin, pH 7.4, made up in filtered, UV-treated dH_2O) on ice. Brains were homogenised with 10 – 15 strokes of a Potter homogeniser. The homogenate was transferred into two 50 ml Falcon tubes (approximating 4 brains per tube) and centrifuged at 500 g for 5 min (Eppendorf Centrifuge 5804R). The

supernatant was removed and discarded and pellets were resuspended in 10 ml 70% Percoll. A Percoll gradient was made by overlaying 10 ml 30% Percoll on top of the 70% Percoll, and finally 10 ml 1xPBS (Fig. 2.1A). The gradients were centrifuged at 1250 g (Eppendorf Centrifuge 5804R) for 50 min with no brake or acceleration. This separated the microglia from the remaining brain matter, and enabled microglia to be collected from the interface between the 70% and 30% Percoll gradient (Fig. 2.1B).

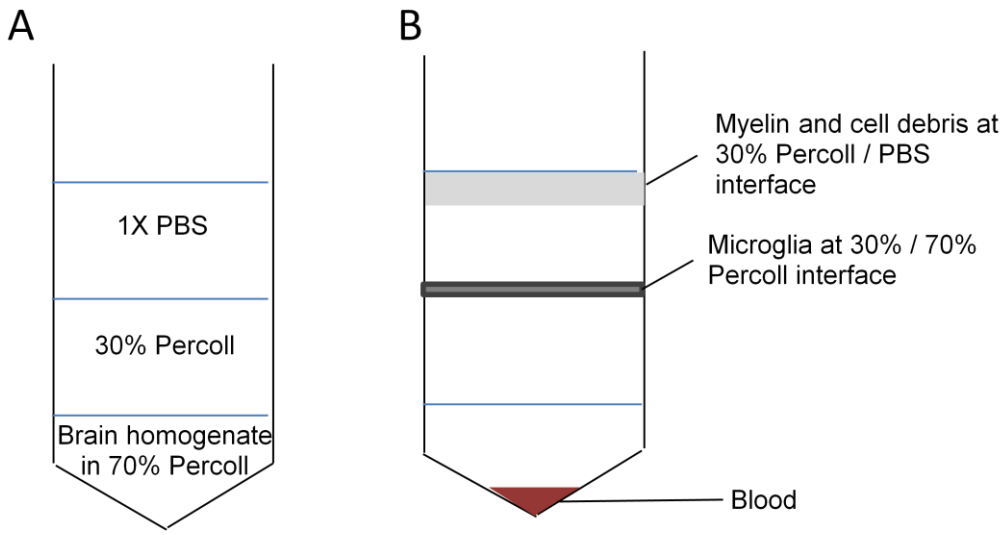

Figure 2.1 Microglial preparation using a percoll gradient. *The brain homogenate is resuspended in 70% percoll, which is overlaid with 30% percoll and finally 1/PBS (**A**). Following centrifugation at 1250 x g for 50 min, microglia are separated from the homogenate and migrate to the 30% / 70% percoll interface, whilst blood pellets at the bottom of the tube, and myelin and cell debris is collected from the 30% / PBS interface (**B**).*

Following removal of the microglia, cells were washed in 50 ml PBS to remove Percoll contamination by centrifugation at 500 g (Eppendorf Centrifuge 5804R) for 5 min. The resulting pellet was then resuspended in 1 ml medium (MEM supplemented with 10% FBS, 20 mM KCl, 30 mM D-glucose, 2 mM L-glutamine, 25 mM $NaHCO_4$, 50 U/ml penicillin, 50 µM streptomycin, and 6 µg/ml ampicillin) and microglia were counted using a

haemocytometer. Microglia were plated on 13 mm glass coverslips at a density of $5x10^4$ cells per well in a 24 well plate. Microglia were initially plated in a small volume of media (100 µl) to allow cells to adhere, which was then increased to 500 µl following 30 min incubation in a humidified incubator with 6% CO_2 at 37ºC. Microglia were left for 24 h before being washed 3 times in medium to remove any debris. The media was then changed to serum-free medium (MEM supplemented with 20 mM KCl, 30 mM D-glucose, 2 mM L-glutamine, 25 mM $NaHCO_4$, 50 U/ml penicillin, 50 µM streptomycin, and 6 µg/ml ampicillin) and microglia were left to rest for at least 3 h before treatment. Microglia were used within 48 h of isolation.

Microglia have been characterized following isolation using the Percoll method. Staining using the microglial specific marker Isolectin B4 (Fig. 2.2) and OX-42 have shown that cultures routinely contain 90% microglia, with the remaining contamination being astrocytes (Kingham et al. 1999). Furthermore, at 2 DIV only 20% of microglia stain positive for ED-1, suggesting a low level of basal activation (Morgan et al. 2004)

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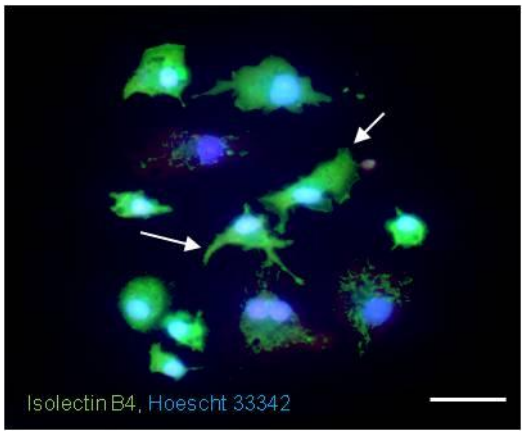

Figure 2.2 Microglia stained with Isolectin B4 and Hoechst 33342. *To confirm the purity of the microglial culture, cells were stained with Isolectin B4 (2 µg/ml), which binds specifically to cells of a monocytic lineage, and Hoescht 33342 (0.6 µg/ml) to counter stain the nuclei. Arrows point to ramified, resting microglia. Cells were imaged using an Axiotop Fluorescence microscope (Zeiss) and a x40 objective. Scale bar 20 µm.*

2.2.1.3 Cerebellar Granule Cell (CGC) culture

CGCs are excitatory neurons found in the granule layer of the cerebellum, which receive excitatory inputs from the mossy fibers and release glutamate (Gallo et al. 1982). In studies in which the neurotoxic or neuroprotective effects of microglia are investigated, CGCs are the predominant neuronal type used, as the cerebellum can be isolated easily and the remaining brain can be used for isolation of microglia.

Cerebellae were collected and CGCs were isolated as described by Courtney et al. (1990). Five day old Sprague Dawley rat pups were killed as described (section 2.2.1.2), and cerebellae were collected on ice into a buffer consisting of 153 mM Na^{+}, 4 mM K^{+}, 1.5 mM Mg^{2+}, 139 mM Cl^{-}, 10 mM PO_4^{2-}, 1.5 mM SO_4^{2-}, 14 mM glucose, and 50 µM BSA (pH, 7.4). This solution is named solution B and will be referred to as such for the remainder of this section. Cerebellae were mechanically dissociated with a sterile razor blade and collected into solution B supplemented with 0.5 mg/ml trypsin for enzymatic digestion of the extracellular matrix. CGCs in trypsin were incubated for 5 minutes at 37°C with gentle agitation every 1-2 minutes. Trypsinisation was neutralized by addition of 20 ml solution B containing 8 µg/mL soybean trypsin inhibitor (SBTI) and 8 U/ml DNAase, the suspension was centrifuged at 65 g for 5 minutes to pellet the CGCs. The supernatant was discarded, and the pellet resuspended and triturated in 3 ml of buffer (solution B containing 3 mM Mg^{2+} and SO_4^{2-} supplemented with 50 µg/mL SBTI and 50 U/ml DNAase) using three fire-polished glass Pasteur pipettes of progressively decreasing diameter. Triturated, homogenous perikarya were layered onto 5 ml 4% BSA in Ca^{2+}-free Earle's Balanced Salts Solution (EBSS) and centrifuging for 5 min at 100 g to remove debris. The pellet was resuspended in warmed CGC medium (MEM supplemented with 10% FBS, 20 mM KCl, 30 mM D-glucose, 2 mM L-glutamine, 25 mM $NaHCO_4$, 50 U/ml penicillin, 50 µM streptomycin, and 6 µg/ml ampicillin) at a density of

$8x10^6$ cells/ml. CGCs were then plated at $8x10^5$ cells per well onto 13 mm diameter poly-Dextro-lysine (PDL)-coated coverslips in a volume of 100 µl media. To give perikarya time to adhere, additional CGC medium (to a total volume of 500 µl) was not added until at least 1 h after plating. After 24 hours, the cells were washed, and half the media was replaced with MEM supplemented with 20 µM cytosine furanoarabinoside (Ara-C) to prevent further glial proliferation. The cultures were maintained at 37°C in a humidified atmosphere of 6% CO_2, and were available for use after 6 DIV.

2.2.2 Cell treatments

2.2.2.1 Treatment of primary and BV2 microglia

Primary and BV2 microglia were routinely cultured for 24 h before use. The medium was changed to serum free medium for at least 3 h before treatment to ensure that cells were down-regulated and in a resting state. Primary and BV2 microglia were treated with LPS, PMA, neurotransmitters, and receptor agonists and antagonists for 24 h after initial time and concentration dependency analysis. Table 2 provides a list of the compounds used, the concentrations and suppliers, and also solvent controls. The concentrations listed are the final concentrations used, as determined by titration assays, or from the literature.

Compound	Description	Concentration	Supplier
PMA	PKC activator	10 ng/ml	Sigma 79346
LPS	TLR4 agonist and iNOS inducer	1 μg/ml	Sigma L9404
Glutamate	Excitatory neurotransmitter	1 μM	Sigma G5921
GABA	Inhibitory neurotransmitter	100 μM	Sigma A2129
BzATP	Excitatory neurotransmitter	250 μM	Sigma B6396
L-AP4	mGluR group III agonist	100 μM	Tocris # 0103
MAP4	mGluR group III antagonist	500 μM	Tocris #0711
MTEP	mGluR group I antagonist	100 nM	Tocris # 2921
AIDA	mGluR group I antagonist	250 μM	Tocris # 0904
CDPPB	mGluR group I agonist	500 nM	Tocris # 3235
DHPG	mGluR group I agonist	100 μM	Tocris # 0342
NAAG	mGluR3 (group II) agonist	50 μM	Sigma A5930
DCG-IV	mGluR group II agonist	500 nM	Tocris # 0975
APICA	mGluR group II antagonist	200 μM	Tocris # 1073
NMDA	NMDA receptor agonist	100 μM	Sigma M3262
MK-801	NMDA receptor antagonist	10 μM	Sigma M107
Quisqualic Acid	AMPA receptor agonist	10 μM	Sigma Q2128
CNQX	AMPA receptor antagonist	10 μM	Sigma C127
Muscimol	$GABA_A$ receptor agonist	50 μM	Tocris # 0298
SKF-97541	$GABA_B$ receptor agonist	100 μM	Tocris # 0379
Picrotoxin	$GABA_A$ receptor antagonist	100 μM	Tocris # 1128
SR95531	$GABA_A$ receptor antagonist	10 μM	Tocris # 1262
UTPγS	$P2Y_{2/4}$ receptor agonist	100 nM	Tocris # 3279
MRS2365	$P2Y_1$ receptor agonist	1 μM	Tocris # 2157
Thioridazine	Nox4 inhibitor	1 μM	Tocris # 3070
Rottlerin	PKCδ inhibitor	10 μM	Tocirs # 1610
Wortmannin	PI3-K inhibitor	1 μM	Tocris # 1232
Apocynin	Nox1/2 inhibitor	10 μM	Calbiochem 178385
PD-98059	p44/42ERK inhibitor	30 μM	Tocris # 1213
SB-203580	p38MAPK inhibitor	10 μM	Tocris # 1202
DMSO	Solvent	<1%	Sigma D4540

Table 2. Compounds used for treatment of BV2 cells and primary microglia.

Following treatment, the conditioned medium was routinely taken from primary microglia, for use in microglial conditioned media (MGCM) studies to investigate the effects of soluble factors on neuronal survival. For this reason, microglia were grown in MEM media to enable neuronal survival. Medium was removed and centrifuged immediately at 10,000 g for 5 min at room temperature (Eppendorf 5415R benchtop centrifuge) to pellet any cell debris, which can affect neuronal activation. The supernatants were then aliquotted and stored at -20ºC until required. To preserve the integrity of the soluble factors, aliquots were defrosted and used only once.

Microglia and BV2 microglia treated as shown in Table 2 were used to assess NADPH oxidase expression, activity and the production of superoxide, in addition to an investigation into the signalling cascades involved in these processes.

2.2.2.2 Treatment of CGCs

CGCs were either treated directly with the compounds described in Table 1 to determine whether they had a direct effect on neuronal survival, or were treated with MGCM from microglia treated as described in Table 1 to determine whether soluble factors released from microglia affected neuronal survival. CGCs subjected to treatment with MGCM were analysed for death using live / dead staining and signalling cascades leading to cell survival or death were analysed by Western blotting.

2.2.2.3 Conditioned media assays

CGCs were treated with MGCM to assess survival following the release of toxic or protective factors from microglia after modulation of the NADPH oxidase, and also to investigate the signalling pathways involved in neuronal survival or death. Microglia were plated in 24 well plates as described and treated as shown in Table 1, and media was retained as described.

During the MGCM assays, half of the medium already on the CGCs (250 µl) was removed from each well, and replaced with 250 µl of MGCM. Only half the media could be removed as CGCs release growth factors which are essential to their survival, therefore removing all of these trophic factors would have a detrimental effect on neuronal survival and may also increase cell death in a way not attributed to the MGCM (Taylor et al. 2002). Following MGCM treatment (which was performed at a number of time points as shown in the relevant results sections), CGCs were either imaged to assess cell death by nuclear morphology and propidium iodide staining, or were lysed to asses signalling pathway activation for analysis by Western blotting.

2.2.3 Cell imaging

Primary and BV2 microglia and CGCs were imaged using a Zeiss Axiotop 2 fluorescence microscope (Oberkochen, Germany). The microscope was used to obtain images of iNOS and ED-1 immunoreactivity of microglia, in addition to the use of nuclear stains to assess cell death, and also for use in the imaging and detection of ROS. All cells were viewed using a 40x Neofluar objective, and images were captured using a Zeiss AxioCam HRc camera and Zeiss Axiovision 3.1 software. Images were analysed using Image J software.

2.2.3.1 Immunocytochemistry

Primary microglia plated on 13 mm glass coverslips in 24 well plates were grown in serum free media before activation for 24 h with the compounds shown in Table 1 (either alone or in combination). After activation, cells were fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature. PFA was then removed, and cells were washed once with PBS, before being permeabilised with methanol for 20 min at -20ºC and washed with PBS three times. Non-specific antigens were blocked using 4% normal goat serum (NGS) for 30 min at room temperature. Cells were then incubated with the primary antibodies (shown in Table 2) diluted in 1xPBS, overnight at 4ºC. The following day, cells were washed in 1xPBS three times and incubated with the secondary antibodies as shown in Table 2 for 2 h at room temperature. Cells were then washed in PBS before being incubated with DAPI (4'-6-Diamidino-2-phenylindole – a nuclear stain that binds to double stranded DNA) 1:1000 at room temperature for 1 min. The cells were again washed in PBS three times, followed by one final wash in distilled water. Coverslips were then mounted on glass slides using Vectashield mountant. Slides were stored away from light at 4ºC until required, and coverslips were viewed and imaged using a Zeiss fluorescent microscope (Zeiss Axioskop 2, Oberkochen, Germany) with tetramethyl rhodamine iso-thiocyanate (TRITC - red fluorochrome, 543 nm), fluorescein isothiocyanate (FITC - green fluorochrome 488 nm) and DAPI (blue fluorochrome 364 nm) filters.

Primary Antibody	Specificity	Dilution	Corresponding secondary antibody	Dilution
Mouse anti ED1 (Serotec MCA275R)	Binds to CR3 receptor in ramified microglia	1:100	Sheep anti mouse IgG FITC (Sigma F2883)	1:1000
Rabbit anti iNOS (BD transduction lab 610333)	Binds to inducible nitric oxide synthase (iNOS)– a marker of activated microglia	1:500	Goat anti rabbit IgG TRITC (Sigma T6778)	1:1000

Table 3. Antibodies used for immunostaining.

2.2.3.2 Assessment of cell death

Neuronal death was assessed by nuclear morphology and the incorporation of propidium iodide (PI) into necrotic and late apoptotic nuclei (Fig. 2.3). During apoptosis, nuclei become smaller and stain brightly with 2´-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5´-bi-1H-benzimidazole (Hoechst 33342) as the chromatin condenses (Kroemer et al. 2009). Hoechst is membrane permeable, and can access the nucleus at all stages of cell survival and death, allowing for assessment of health. The pyknotic nuclei observed with Hoescht staining (Fig. 2.3) are a hallmark of apoptosis and can therefore be counted to determine the percentage of apoptosis in a culture. The necrotic and late apoptotic marker PI is not membrane permeable, and is only incorporated into the nucleus when the nuclear membrane is compromised, which is not seen during early apoptosis and therefore allows for a distinction between different types of cell death (Kroemer et al. 2009).

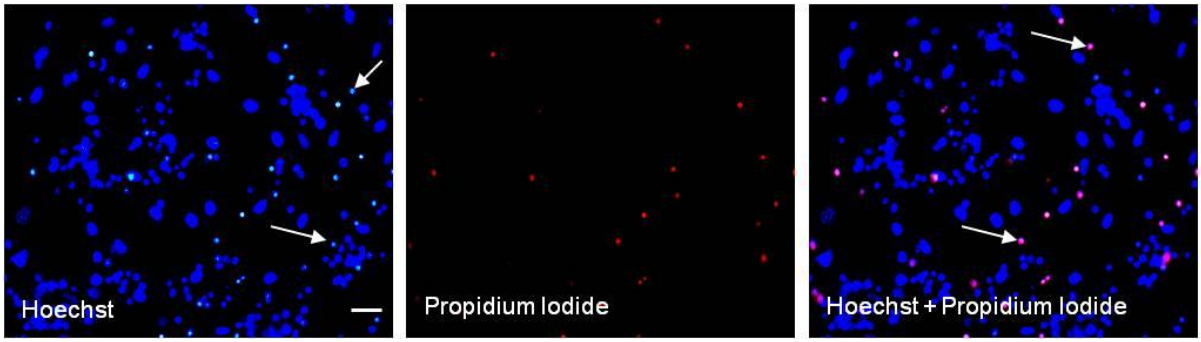

Figure 2.3 Hoechst 33342 and Propidium Iodide live staining of CGC's to assess cell death. *CGC's cultured for 7DIV were stained with Hoechst 33342 (0.6 µg / ml) to show nuclear morphology and propidium iodide (1 µg/ml) to show necrosis. Cells were imaged using a Zeiss axiotop fluorescence microscope and a x40 objective. Images were taken under separate DAPI and TRITC excitation wavelengths and images were merged to show apoptotic and pyknotic nuclei. Arrows in the Hoechst image point to apoptotic nuclei, whilst those in the merged image point to necrotic nuclei. In this way apoptosis could be differentiated from necrosis. Scale bar 20 µm.*

Nuclear staining was performed on live, unfixed CGCs to determine the effects of MGCM on neuronal survival. Following treatments with MGCM, cells were incubated with PI (1 µg/ml) for 30 minutes and Hoechst 33342 (0.6 µg/ml) for 20 minutes. After incubation, the live cells were mounted in basic medium (3 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, 1.2 mM Na_2SO_4 and 1.3 mM $CaCl_2$ at pH 7.4) and were viewed using the TRITC and DAPI filters (Fig. 2.3). Three fields per coverslip were imaged, and the total number of cells and PI positive cells in each field were counted to enable the calculation of the percentage of dead cells (PI stained and pyknotic nuclei). Cell counts were performed using Image J software.

2.2.4 Measurement of superoxide production

Throughout this thesis, four methods have been used for the detection of superoxide and ROS in BV2 and primary microglia: The nitro-blue tetrazolium chloride (NBT) reduction assay; detection of superoxide production by dihydroethidium (dHEth) using fluorescence imaging or flow-cytometry; and high performance liquid chromatography (HPLC) of dHEth oxidation products, to detect the relative production of superoxide and hydrogen peroxide.

There have been many studies investigating and validating the use of exogenous probes and methodologies for ROS detection. It was therefore important to use a variety of probes and techniques in this thesis, so that data could be compared and to ensure that the effects observed were not artefacts of the experimental technique. There are several compounds that can be used to detect superoxide production, however as the focus of this study is on the production of intracellular superoxide as a signalling molecule; the two compounds used to investigate this were NBT, which is reduced to the blue formazan precipitate $NBTH_2$ by superoxide, and dHEth, which is oxidised to 2-hydroxyethidium (2-OH-E^+) by superoxide, and to ethidium (Eth) by hydrogen peroxide.

Whilst shown to be effective in studying the production of superoxide, the use of probes that are oxidised or reduced can produce artefacts. Probes that exploit the oxidative activity of superoxide, such as dHEth, can be oxidised by other ROS such as hydrogen peroxide, peroxynitrite, the thiyl radical and other haeme compounds (Tarpey et al. 2004; Dikalov et al. 2007). Because of this, dHEth may show a lower specificity to superoxide than probes which are reduced by superoxide (Janiszewski et al. 2002), such as NBT. However, probes that are reduced by superoxide can undergo redox cycling which is caused by the reaction of molecular oxygen with partially reduced probe, which can itself generate superoxide, resulting in false positive results (Vásquez-Vivar et al. 1998; Spasojevic et al. 2000). Therefore the use of probes specific to the oxidising potential of superoxide reduces the generation of false-positive readings, whilst the use of probes exploiting the reductive chemistry of superoxide may be more specific (Laurindo et al. 2008). In this thesis, both probe types were used to validate findings.

In this thesis, the specificity of ROS production by the NADPH oxidase was assessed by conducting all experiments both in the presence of the activating stimuli alone and also in the presence of the activating stimuli plus apocynin, an inhibitor of the NADPH oxidase which

prevents the assembly of the enzyme by binding to the gp91phox binding site on p47phox and inhibiting translocation and assembly of the active NADPH oxidase complex, thus preventing superoxide production (Stefanska & Pawliczak 2008). This therefore reduced the incidence of false positive results. Furthermore, the use of HPLC enabled the determination of the reduction of dHEth by both superoxide and hydrogen peroxide, enabling the investigation into which oxidising species was most prevalent following treatment with different activating stimuli. Using these methods, the production of ROS could be attributed to the functionality of the NADPH oxidase.

2.2.4.1 NBT detection of intracellular superoxide

NBT has been used to assess the production of superoxide both *in vivo* and *in vitro* since its discovery as a superoxide scavenger (Baehner et al. 1976; Van Noorden & Butcher 1989). NBT is converted from a yellow liquid to a blue formazan precipitate following exposure to the superoxide anion (Fig. 2.4), which enables quantification of superoxide production *in vitro* by analysis of the absorbance of the formazan precipitate at 630 nm (Choi et al. 2006). The specificity of NBT for superoxide has been demonstrated in studies using SOD, which converts superoxide to hydrogen peroxide. NBT reduction was diminished by 60% after addition of SOD to PMN cells, suggesting that the majority of NBT is reduced by superoxide rather than hydrogen peroxide (Baehner et al. 1976).

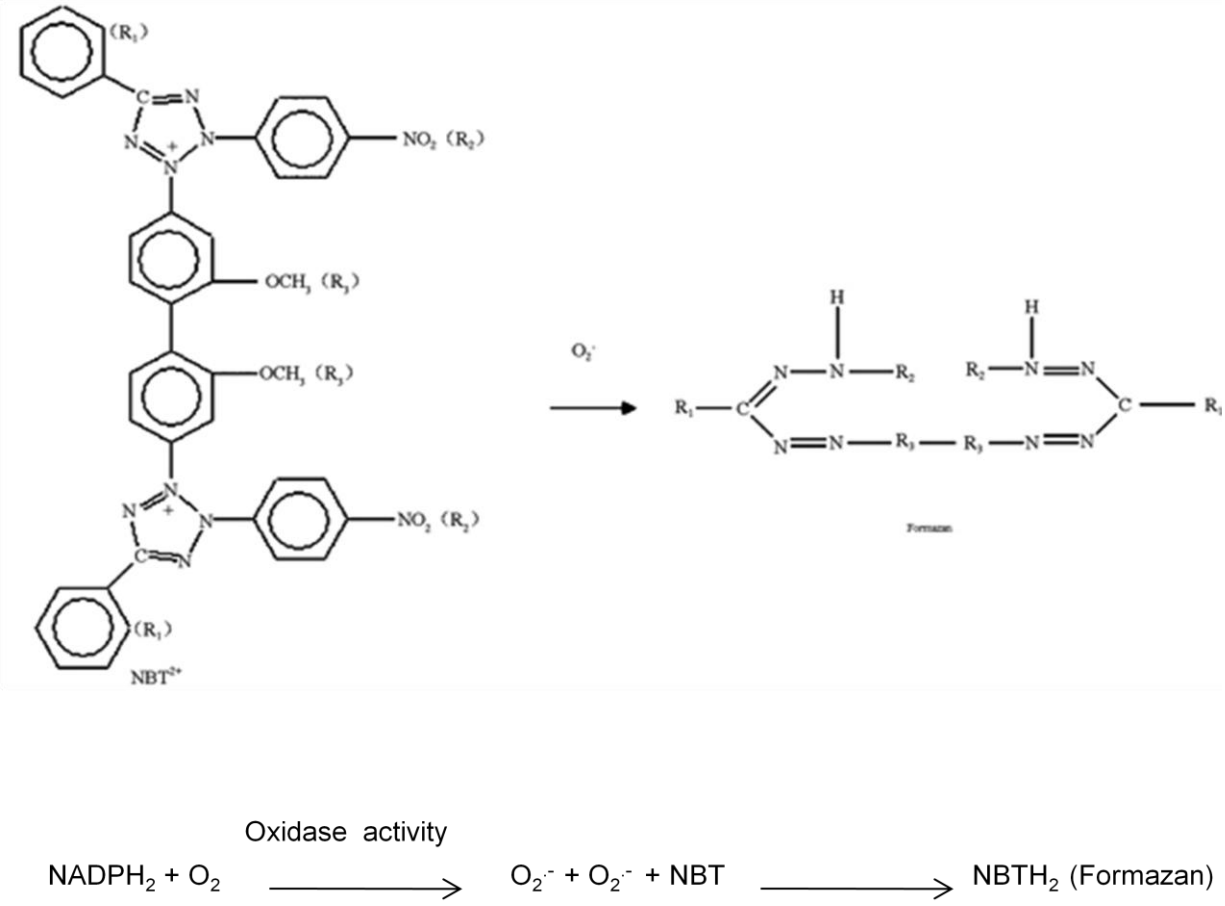

Figure 2.4 Reduction of NBT to $NBTH_2$ formazan precipitate. *Yellow NBT is reduced by superoxide to the blue $NBTH_2$ formazan precipitate which can be detected by absornance at 630 nm.*

The reduction of NBT by superoxide is not isolated to the NADPH oxidase system, however in this thesis it has been used to investigate NADPH oxidase activity by co-treating cells with activating stimuli in the presence and absence of the NADPH oxidase inhibitor apocynin. The reduced NBT here is localised within the cell, enabling the assessment of intracellular superoxide production.

2.2.4.1.1 NBT assay optimisation

The NBT assay was optimised from published methodologies (Choi et al. 2006; Serrander et al. 2007). BV2 microglia were plated in 96 well plates at $1x10^4$ cells per well, and were grown to 70% confluency, after which, medium was changed to serum and phenol red free media for 3 h before treatment. During the initial optimisation, cells were treated with PMA which is a known activator of the NADPH oxidase, shown to induce phosphorylation of the p47phox subunit through PKC activation (Tauber et al. 1989); or LPS, a known microglial activator (Andersson et al. 1992). Initially, an optimum time-point for the detection of intracellular superoxide was determined (Fig. 2.5). Cells were treated with 10 ng/ml PMA (Cox et al. 1985; Kapus et al. 1992) or 1 μg/ml LPS (Taylor et al. 2005) in the presence of 10 μg/ml NBT (Choi et al. 2006) for 30 min, 1 h, 4 h, 8 h, 16 h and 24 h, and NBT reduction was measured by absorbance at 630 nm at each time point using a Tecan x fluo4 plate reader. It was important that formazan precipitate production in BV2 cells treated with the positive control PMA or the microglial activator LPS was significantly increased when compared with basal superoxide production in untreated BV2 cells incubated with NBT only. It was determined that BV2 cells treated for 4 h with PMA or LPS induced a significant increase in superoxide production when compared with basal superoxide levels (Fig. 2.5).

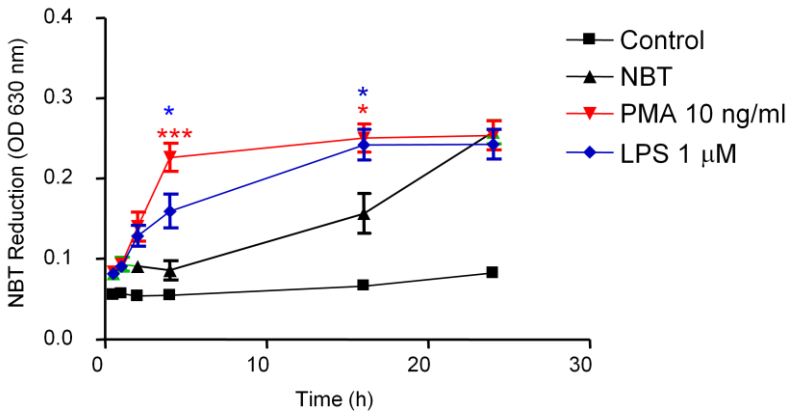

Figure 2.5 Time course analysis of superoxide production by NBT reduction. *BV2 cells plated at 1x10^4 cells per well were incubated in serum and phenol-red free media before treatment with PMA (10 ng/ml) or LPS (1µg/ml) for 30 min, 1 h, 4 h, 8 h, 16 h and 24 h. in the presence of 10 µg /ml NBT. Control cells were incubated with NBT only to detect basal superoxide production. NBT reduction to a blue formazan precipitate was measured at each time point at 630 nm, and was compared to the reduction of NBT in un-treated cells (basal superoxide production). At 4 h incubation there was a significant increase in NBT reduction when compared to cells incubated with NBT only. Significance was determined using a one way ANOVA with Tukey post-hoc analysis at each time-point. ***p<0.001, **p<0.01 and *p<0.05. Data is n=3.*

Following the finding that 4 h treatment with PMA or LPS induced a significant increase in NBT reduction and therefore superoxide production, it was important to determine the optimal concentration of PMA and LPS required to induce superoxide production in BV2 microglia (Fig. 2.6). Cells prepared as described previously were treated with PMA at 1 ng/ml, 10 ng/ml or 100 ng/ml (Fig. 2.6A); or LPS at 100 ng/ml, 1 µg/ml or 10 µg/ml (Fig. 2.6B) in the presence of NBT for 4 h, and NBT reduction was measured at 630 nm. The levels of NBT reduction were compared to cells incubated with NBT only (basal superoxide production). PMA significantly increased NBT reduction at 10 ng/ml, whilst LPS significantly induced NBT reduction at all concentrations (Fig. 2.6A,B).

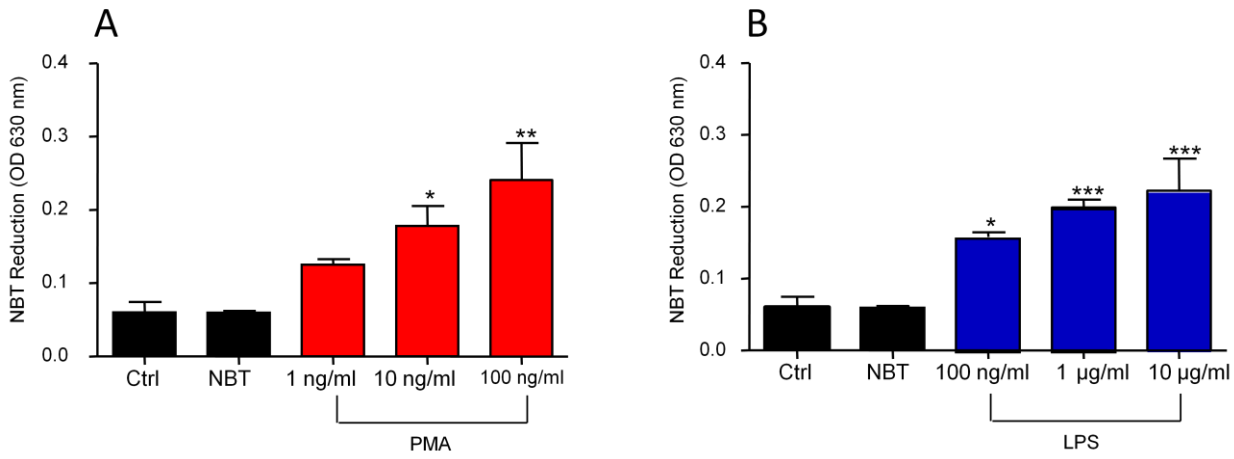

Figure 2.6 Concentration titration of PMA and LPS. *The optimum concentration of PMA and LPS required to significantly reduce NBT when compared to basal superoxide production was determined. BV2 cells were plated at 1x10^4 cells / well and were then incubated in serum and phenol red free media. Cells were then treated with PMA (**A**) at 1 ng/ml, 10 ng/ml or 100 ng/ml; or LPS (**B**) at 100 ng/ml, 1 µg/ml or 10 µg/ml in the presence of 10 µg/ml NBT. NBT reduction was measured after 4 h at 630 nm. Statistical analysis was performed using a one way ANOVA with Tukey post-hoc analysis. PMA or LPS induced increases in NBT reduction were compared with untreated cells incubated with NBT alone. ***p<0.001, **p<0.01 and *p<0.05. Data are n=3.*

It was next important to determine whether PMA or LPS induced superoxide production was a consequence of NADPH oxidase activation, to ensure that this assay was appropriate for the measurement of superoxide production by the NADPH oxidase. Cells were treated with the optimal concentrations of PMA (10ng/ml) or LPS (1 µg/ml) for 4 h, in the presence of the NADPH oxidase inhibitor apocynin, and 10 µg/ml NBT. A concentration titration was performed to determine the optimal concentration of apocynin and its ability to inhibit the reduction of NBT in the presence of PMA or LPS. Cells were co-treated with PMA (Fig. 2.7A) or LPS (Fig. 2.7B) and apocynin at 100 nM, 1 µM, 10 µM or 100 µM. It was shown that apocynin at 10 µM significantly decreased superoxide production induced by PMA or LPS.

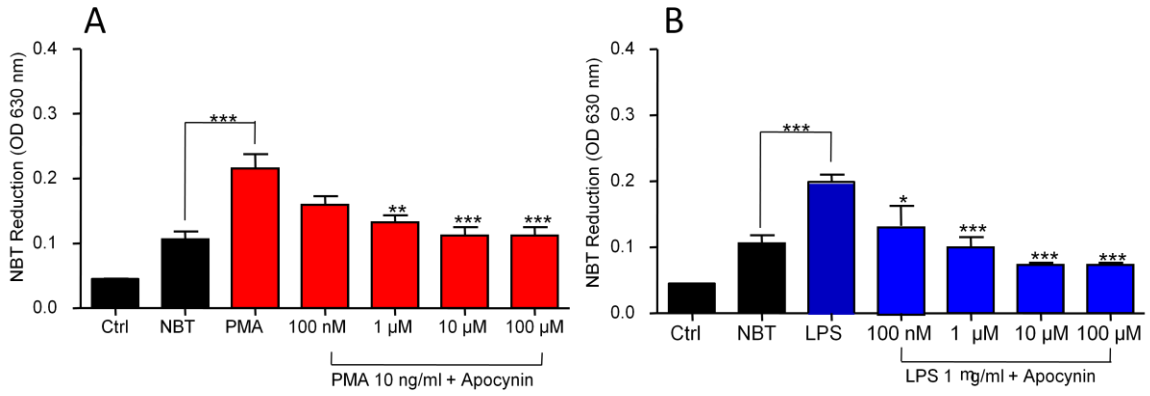

Figure 2.7 Concentration titration of apocynin. *To determine the optimum concentration of apocynin required to inhibit NBT reduction following PMA (**A**) or LPS (**B**) treatment, BV2 cells plated at 1x10^4 cells / well were co-treated with PMA or LPS and apocynin at 100 nM, 1 µM, 10 µM, and 100 µM in the presence of NBT 10 µg/ml and were compared to cells treted with NBT alone for 4 h. To assess statistical significance, a one way ANOVA was performed with Tukey post-hoc analysis. NBT reduction induced by PMA or LPS alone was compared to basal NBT reduction (NBT treated cells only), whilst NBT reduction from co-treatments ie. PMA / LPS plus apocynin was compared to PMA / LPS induced NBT reduction. ***p<0.001, **p<0.01 and *p<0.05. Data are n=3.*

Superoxide production induced by PMA or LPS was significantly attenuated by apocynin, suggesting activation of the NADPH oxidase. To ensure that these data were not a result of an increase in proliferation or toxicity of apocynin, cell counts were performed on BV2 cells plated in 24 well plates on glass coveslips at 2.5x10^4 cells per well. Cells were stained with the nuclear dye Hoechst 33342 (0.6 µg/ml), as described previously, and cells were counted. Fig. 2.8 shows that apocynin alone or in combination with PMA (Fig. 2.8A) or LPS (Fig. 2.8B) does not induce cell death, and PMA or LPS treatments do not induce proliferation.

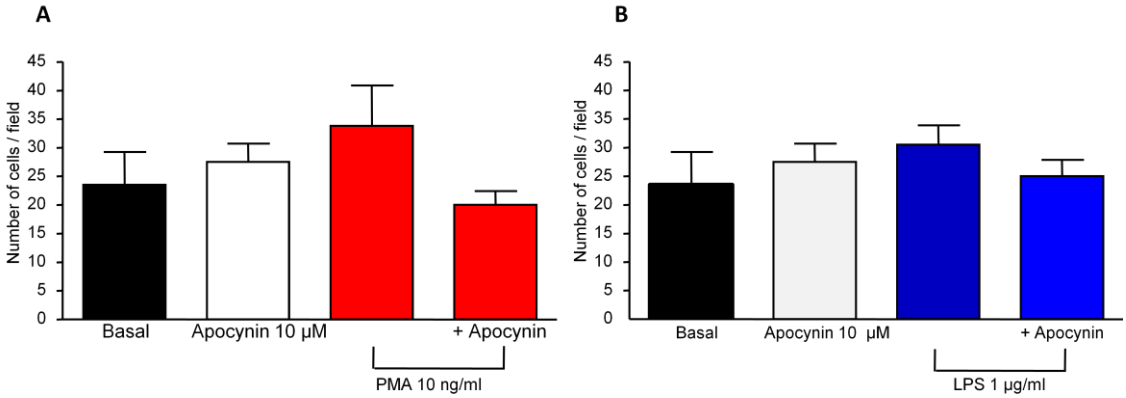

Figure 2.8 PMA, LPS or Apocynin toxicity. *BV2 cells were plated in 24 well plates at 2.5x10^4 cells / well and were either un-treated, or treated with apocynin 10 µM, PMA 10 ng/ml, LPS 1 µg/ml or apocynin + PMA (**A**) or apocynin + LPS(**B**). Statistical analysis was performed using a one way ANOVA with Tukey post-hoc analysis. There was no difference in cell number across the conditions. Data are n=3.*

These optimisation studies showed that NBT is a suitable probe for the detection of superoxide produced by the positive controls PMA and LPS, and that the assay could be used to measure superoxide production as a consequence of NADPH oxidase activation, as NBT reduction following treatment with PMA or LPS could be inhibited by apocynin (Fig. 2.9).

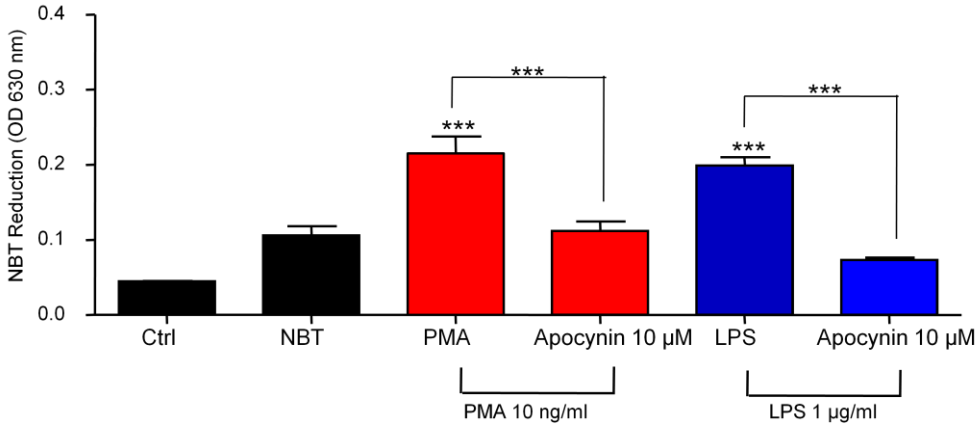

Figure 2.9 Optimal conditions for the detection of superoxide production using the NBT assay. *BV2 cells treated with 10 ng/ml PMA or 1 µg/ml LPS in the presence of NBT for 4 h induced a significant increase in NBT reduction (superoxide production) when compared to basal superoxide production (cells incubated with NBT alone). Co-treatment with PMA and apocynin or LPS and apocynin significantly reduced superoxide production when compared to single treatments, suggesting that PMA and LPS induce activation of the NADPH oxidase. Statistical analysis was performed by one way ANOVA, and Tukey post-hoc analysis was used. ***p<0.001, **p<0.01 and *p<0.05. Data are n=3.*

2.2.4.1.2 Optimised NBT assay protocol

BV2 microglia were plated in 96 well plates at a density of $1x10^4$ cells per well. Cells were grown for 24 h before media was changed to serum and phenol red free media for 3 h before the assay. Cells were then treated with the compounds shown in Table 1 (either alone or in combination with apocynin) in triplicate in phenol-red and serum free media containing 10 μg/ml NBT. To assess basal superoxide production, and as a control for all comparisons, untreated cells were incubated with NBT alone. Cells were incubated for 4 h (in agreement with Jekabsone et al. 2006; Serrander et al. 2007) at 37ºC in a humidified atmosphere with 6% CO_2. The optical density of the formazan precipitate was measured by absorbance at 630 nm using a plate reader (Tecan x fluo4 plate reader). Experiments were performed using three separate cell preparations and were conducted in triplicate.

2.2.4.2 Dihydroethidium detection of intracellular superoxide – assessment by fluorescence microscopy

Dihydroethidium (dHEth) is a superoxide sensitive probe used for the detection of intracellular superoxide. It is a hydrophobic, uncharged molecule that can readily cross the plasma membrane and can enter membrane bound organelles (Garbett et al. 2004). Upon oxidation, dHEth becomes positively charged and is oxidised to 2-OH-E^+, which accumulates in the nucleus of cells producing superoxide where it intercalates into the DNA through electrostatic interactions between the phosphate groups (Garbett et al. 2004) and hydrophobic interactions between the ethidium moiety and the minor groove of the DNA double helix (Jain et al. 1977; Garbett et al. 2004) and fluoresces red (excitation 490, emission 590). Furthermore, this red fluorescence is obtained more readily with superoxide generating systems, such as the xanthine and glucose oxidase (Benov et al. 1998), over oxidants such as hydrogen peroxide, peroxynitrite or the hydroxyl radical (Bindokas et al. 1996). In support

of this, there have been many reports suggesting that superoxide is the only biologically relevant oxidant that reacts with dHEth to form 2-OH-E^+ (Fig. 2.10), making this a highly specific marker for superoxide (Zhao et al. 2003; Robinson et al. 2006; Zielonka et al. 2008).

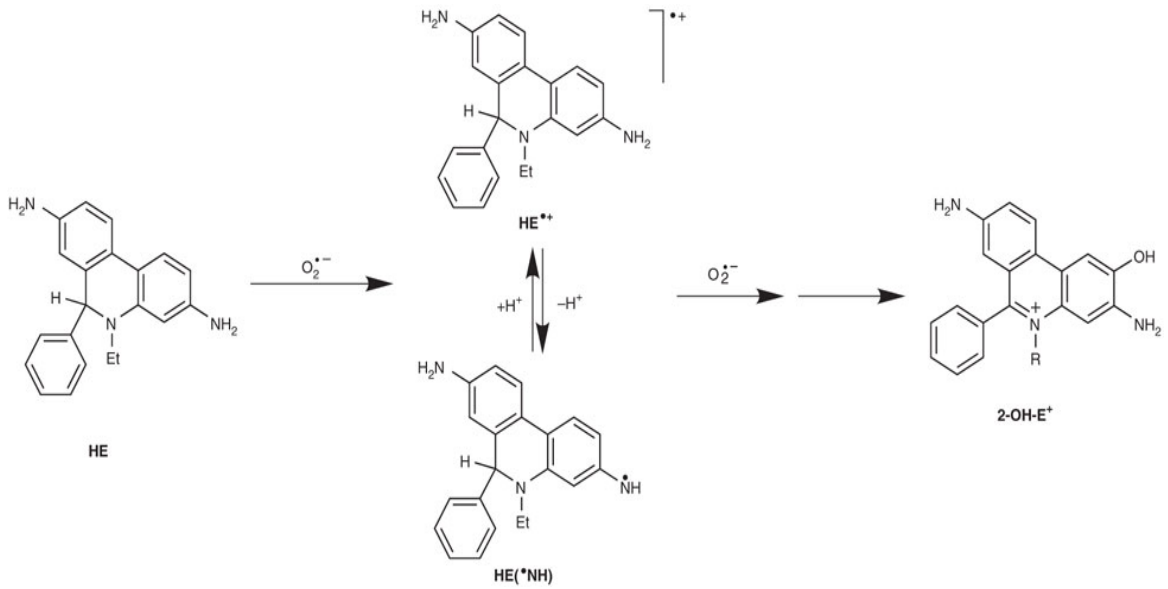

Figure 2.10 The oxidation of dihydroethidium to 2-OH-E^+ by superoxide. *Dihydroethidium (HE) is converted to 2-OH-E^+ in a step- wise manner. HE is oxidized by superoxide to produce two radical species $HE^{\cdot-}$, and HE(˙NH). These intermediates react rapidly with another superoxide anion to give hydroperoxide, which forms the imino-quinone derivative of HE upon water elimination, and then re-arranges to form 2-OH-E^+. (Figure from Zielonka et al. 2008).*

The oxidation of dHEth by superoxide proceeds in a two step manner (Fig. 2.10). The radical formed in the first step reacts rapidly with another superoxide anion to form hydroperoxide, which forms an imino-quinone derivative of dHEth upon elimination of water, and rearranges to form 2-OH-E^+ (Zielonka et al. 2008). The specificity of dHEth for superoxide therefore makes it an attractive probe for the detection of superoxide production *in vitro* (Robinson et al. 2006; Dikalov et al. 2007). Furthermore, the fluorescence profile of 2-OH-E^+ enables the use of this probe for imaging using fluorescence microscopy as described in this thesis.

2.2.4.2.1 Optimisation of dHEth detection of intracellular superoxide by fluorescence microscopy

Here, the imaging of intracellular dHEth fluorescence was used as an alternative assay to assess intracellular superoxide production in primary microglia (Zhao et al. 2003). The assay was optimised from published protocols (Bucana et al. 1986; Zhao et al. 2003; Larsen et al. 2009). Microglia were plated on glass coverslips in 24 well plates at a density of $5x10^4$ cells per well, and were grown for 24 h in MEM. Media was changed to serum free medium 3 h prior to activation. To optimise the protocol, the known activators of the NADPH oxidase, PMA and LPS were used as positive controls. Microglia were treated with the concentrations deemed to be optimal from the NBT assay (10 ng/ml PMA and 1 μg/ml LPS). Initially, a time course analysis was performed to determine the optimal time after which PMA or LPS induced an increase in the number of superoxide positive cells (cells exhibiting red fluorescence in the nuclei) that was significantly elevated when compared with control untreated cells (Fig. 2.11). Microglia were incubated with PMA or LPS for 30 min, 1 h, 4 h, 8 h, 16 h and 24 h and after each time point, microglia were incubated with dHEth at 5 μM for 40 min (Peshavariya et al. 2007). Microglia were also counterstained with Hoechst 33342 (0.6μg / ml) for 20 min, and coverslips were mounted in basic medium then imaged using the Zeiss fluorescent microscope (Zeiss Axioskop 2, Oberkochen, Germany) with excitation wavelength at 550 nm and emission wavelength at 580 nm for dHEth and excitation at 343 nm and emission at 483 nm for Hoechst 33342. Three fields were imaged per condition and the experiment was performed in triplicate. Microglia were considered superoxide positive when the nucleus fluoresced red, and these microglia were counted along with the total number of cells to enable the calculation of the percentage of superoxide positive cells.

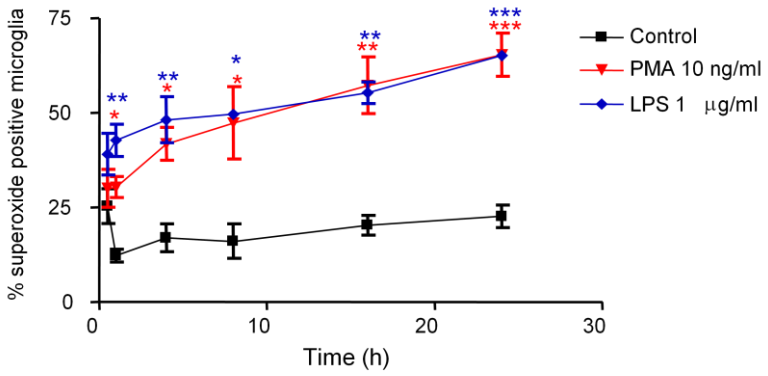

Figure 2.11 Time course analysis of dHEth fluorescence. *To determine the optimal time for incubation of microglia with NADPH oxidase activators to allow the detection of superoxide by dHEth fluorescence, microglia plated on coverslips in 24 well plates were incubated with PMA 10 ng/ml or LPS 1 µg/ml for 30 min, 1 h, 4 h, 8 h, 16 h, and 24 h. Cells were imaged by dHEth fluorescence and were counterstained with Hoechst 3342. Total cell number and superoxide positive cells were counted and percent superoxide positive cells were calculated. Statistical analysis was by one way ANOVA with Tukey post hoc analysis. Comparisons were made between PMA or LPS treated cells and controls. ***p<0.001, **p<0.01 and *p<0.05. Data are n=3.*

From the time-course analysis (Fig. 2.11) 24 h incubation with the activating stimuli induced the most significant increase in the number of superoxide positive microglia. This time point was used for all further experiments.

It was important to determine whether dHEth was appropriate for the detection of superoxide production as a consequence of NADPH oxidase activation. Microglia were therefore treated with PMA (10 ng/ml) or LPS (1 µg/ml) for 24 h in the presence of apocynin (10 µM) (Fig. 2.12) before incubation with 5 µM dHEth for 40 min and 0.6 µg/ml Hoescht 33342 for 20 min before coverslips were mounted in basic medium and imaged as described (Fig. 2.12B). Apocynin significantly reduced the number of PMA and LPS induced superoxide positive cells (Fig. 2.12A and B), showing that this assay was appropriate for the measurement of superoxide production by the NADPH oxidase in microglia.

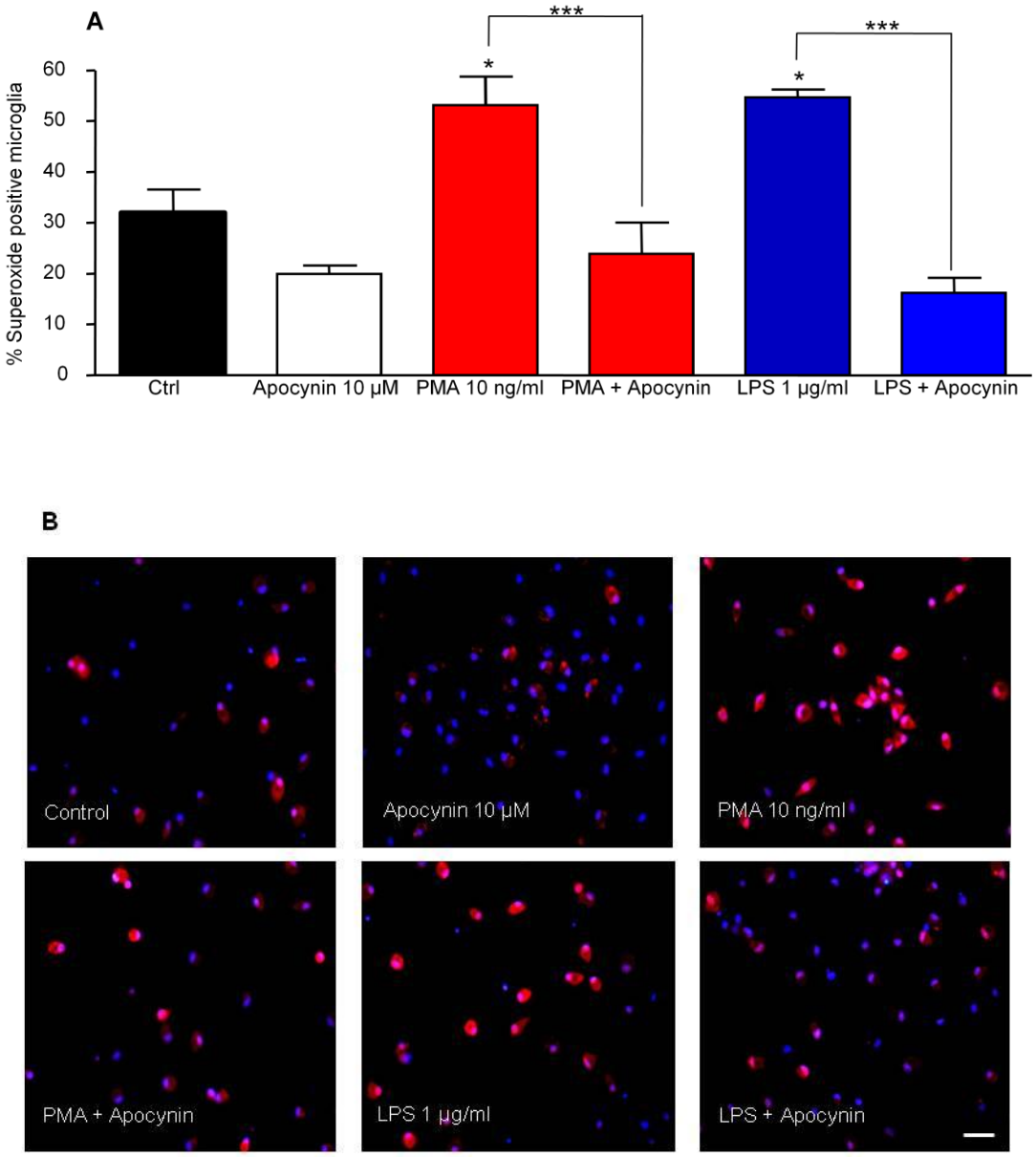

Figure 2.12 Superoxide production in microglia treated with PMA or LPS in the presence and absence of apocynin can be detected by dHEth floursecence. *Microglia plated in 24 well plates were activated with PMA (10 ng/ml) or LPS (1 µg/ml) in the presence or absence of apocynin (10 µM) for 24 h. Microglia were then incubated with the superoxide sensitive probe DHEth (5 µM) for 40 min and were counterstained with Hoechst 3342 (0.6 µg/ml) for 20 min. Superoxide positive cells were determined by red fluorescence in the nuclei as shown (**B**), which decreased upon treatment with apocynin. Superoxide positive cells were counted and a percentage was calculated (**A**). Statistical analysis was performed using a one way ANOVA with Tukey post-hoc analysis ***p<0.001, **p<0.01 and *p<0.05. This showed that superoxide production induced by 24 h incubation with PMA or LPS could be reduced by inhibition of the NADPH oxidase. Data are n=3.Scale bar 20 µm.*

2.2.4.2.2 Optimised dHEth detection of intracellular superoxide by fluorescence microscopy protocol

From these initial experiments, an optimised protocol was developed. Primary microglia were plated on glass coverslips at $5x10^4$ cells per well and were grown for 24 h in MEM. Medium was changed to serum free medium for 3 h before treatment as described in Table 1 either alone or in combination with apocynin for 24 h (as determined by the time and concentration titrations). After this time, live, unfixed cells were assessed for superoxide production. Microglia were treated with 5 μM dHEth for 40 min (Zhao et al. 2003), and were co-stained with Hoechst 33342 0.6 μg/ml for 20 min. Cells were then mounted in basic medium and were imaged using the Zeiss fluorescent microscope (Zeiss Axioskop 2, Oberkochen, Germany) with excitation wavelength at 550 nm and emission wavelength at 580 nm for dHEth and excitation at 343 nm and emission at 483 nm for Hoechst 33342. Three coverslips were imaged per condition, with 6 fields of view taken per coveslip. Experiments were performed in triplicate, using three separate microglial preparations. Images were analysed for superoxide production by counting the number of microglia in which dHEth had intercalated into the nucleus which indicated the oxidation of dHEth to 2-OH-E^+ by superoxide. Some cells expressing high levels of superoxide exhibited high background fluorescence where the 2-OH-E^+ had intercalated into the mitochondrial DNA, which gave the appearance of non-specific binding. These cells were counted so long as the nucleus exhibited red fluorescence. The total cell number was counted, and data were expressed as a percentage of superoxide positive microglia.

2.2.4.3 Flow cytometry analysis of superoxide production

As the detection of superoxide using dHEth fluorescence imaging is a semi-quantitative analysis of superoxide levels within the cell, flow cytometry was used to quantify superoxide production. As mentioned previously, dHEth is converted to the superoxide specific product 2-OH-E^+ upon exposure to superoxide, which intercalates into the nucleus of superoxide producing cells and fluoresces red. This property of dHEth was exploited to count the number of superoxide positive cells within a sample using FACS (fluorescence activated cell sorting) analysis. Flow cytometry relies upon the movement of cells in suspension past a detector, which can measure cell size and granularity, and also the fluorescence of the cell if fluorescent markers have been used. The prepared sample in suspension is taken up from a FACS tube under pressure and transported to the flow cell, where the sample combines with a faster flowing sheath fluid which transports the sample to a light source for excitation, where light scatter and fluorescence are captured and detected by photodetectors. The flow cytometer is able to count single cell events by passing the cell suspension through a narrow tube at high pressure, which forces the cell suspension to pass the laser and detector in an orderly stream of particles. To count the events, the flow cytometer relies on the ability of cells to scatter light if not tagged with a fluorescent probe, or to emit light if tagged with a fluorescent probe. Light is collected by the forward and side collection lenses, which provides the forward scatter (FSC) and side scatter (SSC) data. These refer to cell size and granularity respectively, which can provide information on morphology and the health of the cell. The forward scatter and side scatter of light is passed onto a detector which generates an electrical pulse. When the electrical pulse exceeds the threshold limit, an event is counted and analysed by the acquisition software.

2.2.4.3.1 Optimisation of flow cytometry

As large numbers of cells were required for flow cytometry, BV2 microglia were used in place of primary microglia. As dHEth is used to asses superoxide production in live adherent cells, it was first important to determine whether live BV2 microglia could be used for this protocol, and to ensure that the cells remained viable and did not alter significantly in morphology following detachment from the culture plates, which was required to generate a cell suspension for analysis. Initial optimisation used live, unlabelled cells, detached from the culture flasks using accutase. BV2 microglia were grown in 6 well plates at $10x10^4$ cells per well and were grown to confluencey. The media was changed 3 h before FACS analysis to serum free DMEM, which prevented proliferation and ensured that all cells were in the same stage of the cell cycle to limit variations in cell morphology and size, and to generate a homogenous population for analysis. The medium was aspirated from cells, which were washed once with PBS. Accutase (100 µM) was added to the cells, which were returned to the incubator for 1 min. After this time, cells had detached, and the activity of accutase was stopped by the addition of an equal volume of serum containing medium. Cells were transferred to a 15 ml Falcon tube and pelleted by centrifugation at 3645 g for 5 minutes (Eppendorf Centrifuge 5804R). Cells were then resuspended in 2 ml ice-cold PBS and were kept on ice until analysis. For the analysis of cell viability and morphology, the cell suspension was transferred to a FACS tube (BD Falcon 5 ml round bottomed tube), which was placed in the sample injection port (SIP) and then taken up into the flow cytometer (Becton Dickenson FACSCalibur) for analysis. 30,000 events were counted, based on the scattering of light from un-labelled cells which provided information of the cell size (FSC), and granularity or morphology (SSC), and data was analysed using CellQuest software. The cell population routinely seen following FACS analysis of un-labelleed BV2 microglia is shown in Fig. 2.13.

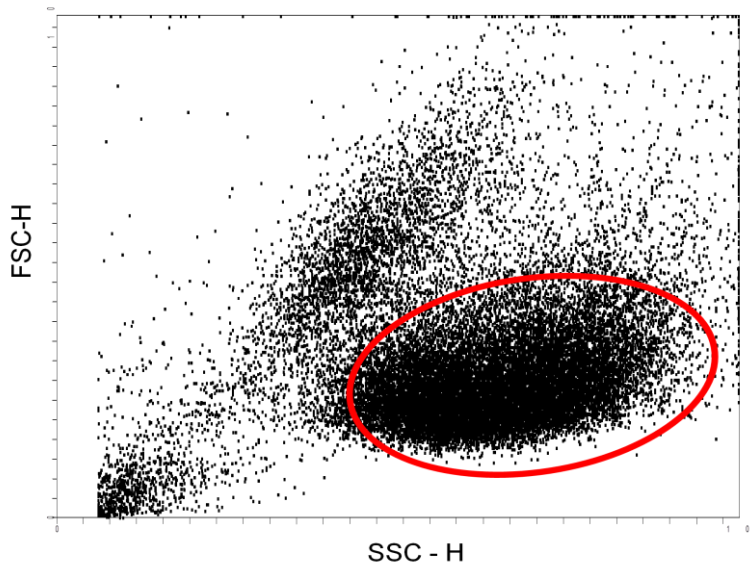

Fig. 2.13 Representative dot-plot from un-labelled BV2 microglia. *The dot-plot shows that BV2 microglia form a distinct homogenous population that have the same size and morphology. The gated population was used in all further analysis as this represented the most homogenous population of BV2 microglia. This therefore enabled BV2 microglia to be used for the FACS analysis of superoxide production in BV2 microglia.*

BV2 microglia were found by FACS analysis to be present in a homogenous population that did not change morphology or size over time, therefore cells grown and prepared in this manner could be used for the detection of superoxide using the dHEth probe.

It was next important to determine that dHEth labelling of BV2 microglia remained stable after cells were detached from their culture wells, and that the fluorescence could be detected by flow cytometry. A method by Hererra et al. (2004) was followed, and to validate the method, the use of flow cytometry to detect superoxide production in BV2 cells treated with the positive control PMA was first tested. BV2 microglia grown to confluency were treated with PMA (10 ng/ml) either alone or in the presence of apocynin (10 µM) in serum free medium for 24 h. Cells were incubated with dHEth (5 µM) for 40 min, before being washed with PBS and detached using accutase. Cells were pelleted and resuspended in PBS (2 ml) and kept on ice before analysis using the FACSCalibur and CellQuest acquisition software,

set to measure fluorescence intensity at 595 nm. A representative histogram and the mean fluorescence intensity from the analysis of dHEth fluoresenece in PMA treated BV2 microglia from three separate cell preparations is shown in Fig. 2.14A, B.

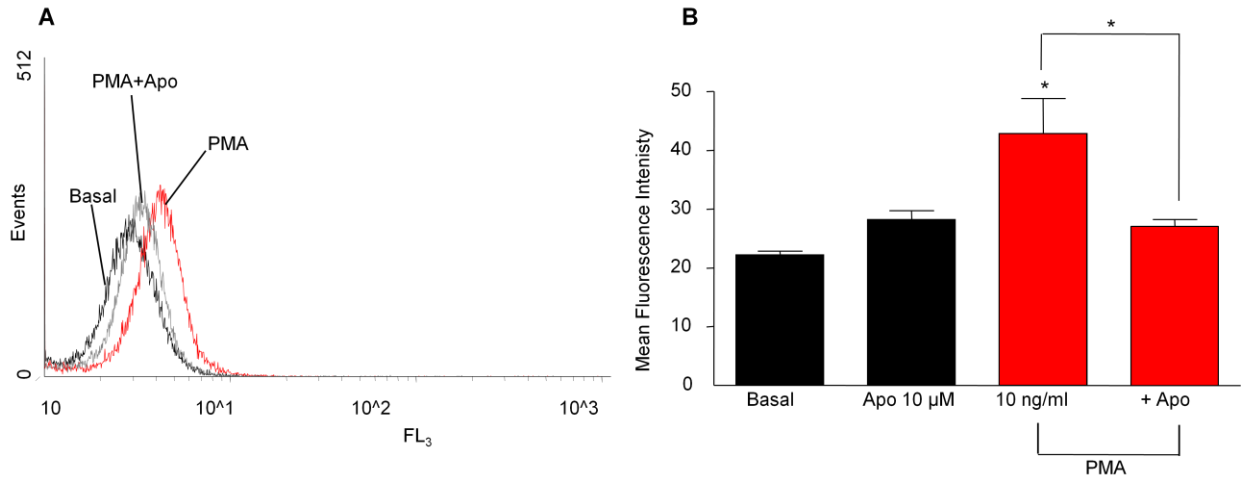

Figure 2.14 FACS analysis of PMA induced superoxide production in BV2 microglia. *BV2 microglia were treated with PMA (10 ng/ml) in the presence or absence of apocynin (10 µM) for 24 h before incubation with dHEth (5 µM) for 40 min and analysis of superoxide production by dHEth fluorescence using flow cytometry. The analysis measured the emission of dHEth fluorescence at 595 nm from 30,000 cells per sample. The experiment was performed three times using three separate cell preparations. Data was analysed by one way ANOVA and Tukey post-hoc analysis, making comparisons between treated cells and un treated cells and also as indicated. *p<0.05. Data are n=3.*

PMA significantly elevated dHEth fluorescence in BV2 microglia, which could be attenuated by apocynin, suggesting that PMA induced superoxide production through activation of the NADPH oxidase. These findings correlated with dHEth imaging by fluorescence microscopy, and showed that flow cytometry was appropriate for the quantitative measurement of superoxide production using dHEth. Furthermore, dHEth labelling remained consistent over three separate experiments, therefore the menthod of labelling and cell preparation was appropriate for FACS analysis of superoxide production.

2.2.4.3.2 Optimised flow cytometry method

To detect superoxide production in BV2 microglia, a protocol from Herrera et al. (2004) was followed. BV2 microglia were plated in 6 well plates at $10x10^4$ cells per well and were grown to confluencey. The medium was changed to serum free medium for 3 h before treatment as shown in Table 1 in the presence or absence of apocynin for 24 h (to maintain consistency with this probe), or cells were left untreated to determine basal superoxide production. Cells were then incubated with 5 µM dHEth for 40 min before dissociation from the culture vessel using accutase. The culture medium was aspirated and cells were washed once with PBS before 100 µl accutase was added to the cells. Cells were returned to the incubator for 1 min and then tapped gently to detach the cells from the plates. Accutase activity was inhibited by the addition of 100 µl serum containing media to the cells, and this cell suspension was collected into a 15 ml Falcon tube and cells were pelleted by centrifugation at 3645 g for 5 min (Eppendorf Centrifuge 5804R). The supernatant was aspirated and the cell pellet was resuspended in 2 ml PBS, transferred to a FACS tube, and kept on ice in the dark until analysis.

Analysis of dHEth fluorescence was measured using a Beckton Dickenson FACSCalibur flow cytometer. For each sample, cells were taken up into the flow cell and excited by a laser at 510 nm, which induced the emission of light at 595 nm from superoxide expressing BV2 microglia labeled with dHEth. 30,000 events were counted and analysed with CellQuest software, which then provided a measure of mean fluorescence intensity for each sample. Each experiment was performed three times using three separate cell preparations to enable the statistical analysis of mean fluorescence intensity for each condition.

2.2.4.4 HPLC detection of superoxide and hydrogen peroxide

In-depth analysis of dHEth oxidation has revealed that it can be oxidised by superoxide but also hydrogen peroxide, to the non-specific product Eth. Furthermore, both 2-OH-E^+ and Eth can intercalate into the nucleus of ROS producing cells, making it difficult to distinguish between superoxide and hydrogen peroxide production by methods other than HPLC (Fink et al. 2004; Laurindo et al. 2008). The fluorescence microscopy analysis of superoxide production used in this thesis (section 2.2.4.2) relies on the fact that superoxide reacts with dHEth to form a fluorescent ethidium product that intercalates within the nucleus of cells. However, as shown here, the production of hydrogen peroxide may produce false positive results, inducing the production of ethidium which also intercalates into the DNA of cells and fluoresces in the red spectrum. The main drawback of the imaging and FACS methods is that they take into account the total fluorescence of all dHEth products, which provides information about the total cell redox state rather than that of specific ROS (Laurindo et al. 2008). With this in mind, and the fact that fluorescently, 2-OH-E^+ and Eth are difficult to distinguish with conventional fluorescence approaches, HPLC analysis was used to determine the relative amounts of 2-OH-E^+ and Eth produced following treatment of cells with the activating stimuli in the presence or absence of apocynin. This technique provides an accurate analysis of the separation and quantification of the superoxide specific product 2-OH-E^+ and the hydrogen peroxide derived product Eth (Fink et al. 2004).

The method for HPLC analysis of dHEth oxidation followed was from Zielonka et al. (2008). HPLC with fluorescence and UV visible absorption was used for the detection of 2-OH-E^+ and ethidium. During HPLC, the mobile phase (50 mM phosphate buffer pH 2.6) is passed through a chromatography column (the stationary phase), which in this case was a Kromasil C_{18} column, under high pressure. The column contains C_{18} chains bonded to microscopic silicon beads, and hydrophobic interactions between the mobile phase and the C_{18} chains

retard the progress of molecules as they are passed through the column. The time taken for a molecule to be eluted from the column (the retention time), depends on its hydrophobicity and is identical under the same conditions. Following elution from the column, molecules are passed through a UV and fluorescence spectrometer and fluorescence detection at 356 nm and 510 nm (excitation) and 595 nm (emission) in addition to absorbance at 220, 250, 290, 370 and 500 nm were used to monitor the reaction products in the sample. Peaks seen following fluorescence excitation at 510 nm and emission at 595 nm corresponded to 2-OH-E^+ and ethidium, depending on the retention time that the peaks were observed at, and the peak areas were measured and compared with the standards to calculate the concentration of the test sample. The retention times for Eth and 2-OH-E^+ were reported to be 15 and 12 min respectively (Zielonka et al. 2008). Ethidium exhibited a significant absorbance peak at 290 nm, with a retention time of 15 min, which could be used to confirm that the peak observed in the fluorescence spectra could be attributed to ethidium (Zielonka et al. 2008).

2.2.4.4.1 Preparation of standards

Standards of 2-OH-E^+, ethidium and dHEth were prepared. Ethidium and dHEth were obtained from Invitrogen and were diluted from stock solutions to genetrate a concentration gradient that could be subjected to HPLC analysis to construct the standard curves for analysis of ethidium and dHEth concentrations in the sample. The synthesis of 2-OH-E^+ depended on the oxidation of dHEth using potassium nitrosodisulfonate (Fremy's salt). To synthesise 2-OH-E^+, a 1 mM stock of Fremy's salt was first prepared. 3.6 g of the Fremy's salt was reconstituted in 10 ml of 50 mM phosphate buffer (pH 7.4) containing 100 µM diethylene triamine pentaacetic acid (DTPA), which promoted the dissociation of the yellow dimeric Fremy's salt to the blue monomeric form of the nitroxide radical, which promotes the oxidation of dHEth to 2-OH-E^+. It was important that the UV-visible absorption spectrum (at 248 and 545 nm) of the dissolved Fremy's salt was recorded to enable a subtraction of any

contaminating Fremy's salt in the final synthesised 2-OH-E^+. The concentration of Fremy's salt was then calculated by:

$C_{cuvette}[mol/dm^3] = A / \varepsilon\ [dm^3\ mol^{-1}\ cm^{-1}] \times l\ [cm]$

Where: *A* is the absorbance values at 248 and 545 nm, ε is the extinction co-efficient value of 1.69×10^3 and 20.8 $dm^3\ mol^{-1}\ cm^{-1}$ at 248 and 545 respectively, and *l* is the optical path length (1 cm).

Following the calculation of Fremy's salt concentration, dHEth was prepared by adding 200 µM dHEth in DMSO to 24 ml of water containing 4 ml of 0.5 mM phosphate buffer pH 7.4 containing 4 ml of 1 mM aqueous DTPA. After mixing, 2-OH-E^+ was generated by adding 8 ml of 1 mM Fremy's salt to the dHEth solution and the reaction was left at room temperature for 2 h. To determine the production and retention time of 2-OH-E^+ the product was analysed by HPLC after dilution in 0.5 mM phosphate buffer pH 2.6 (Fig. 2.15A). HPLC analysis of the ethidium (Fig. 2.15B) and the dHEth (Fig. 2.15C) standards diluted in 0.5 mM phosphate buffer pH 2.6 was also performed to determine purity and to obtain the retention times of these standards.

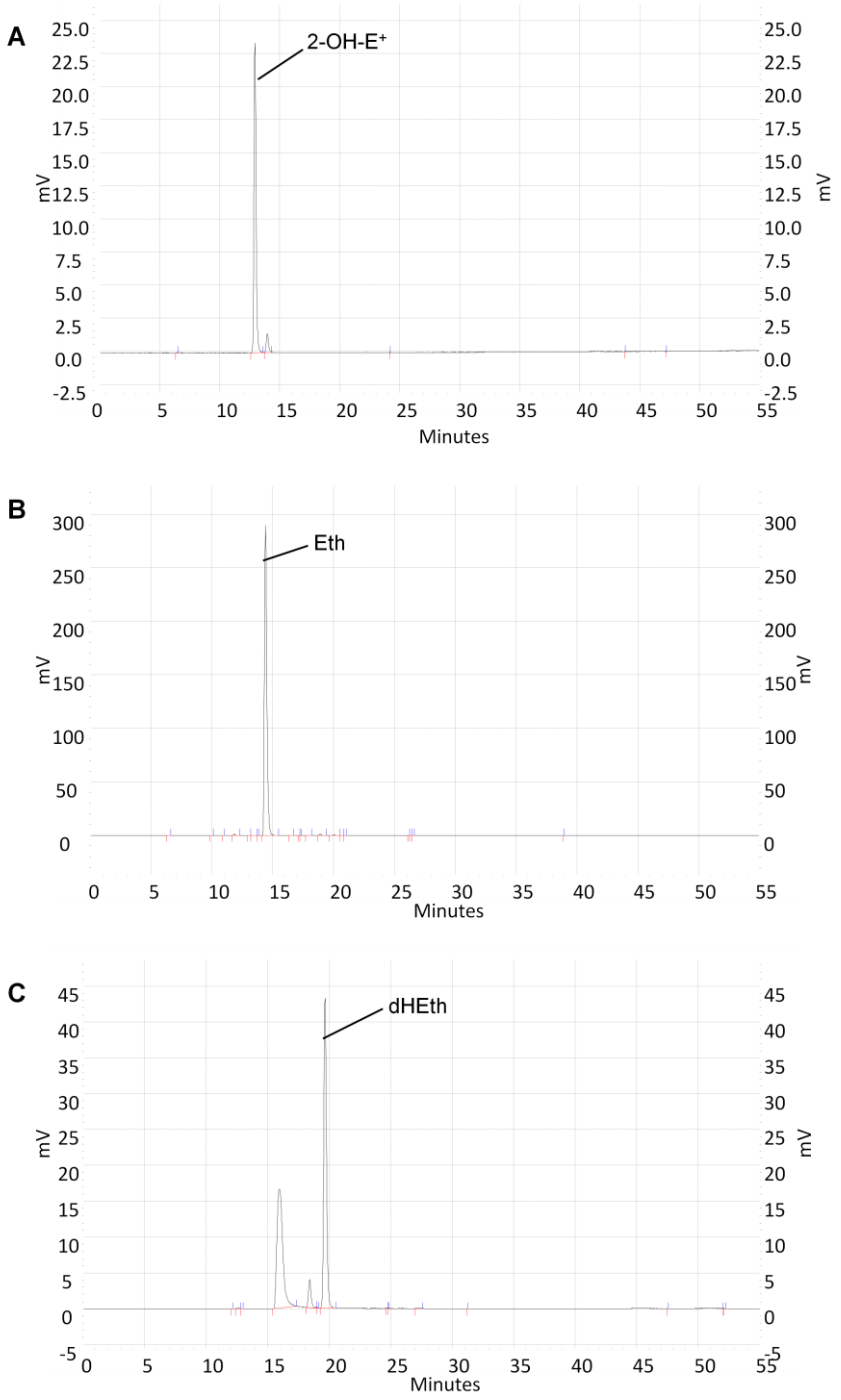

Figure 2.15 HPLC chromatograms of 2-OH-E$^+$ (A), Ethidium (B) and dHEth (C). *Standards of 2-OH-E$^+$, Ethidium and dHEth were subjected to HPLC analysis after dilution to 15 µM in 0.5 mM phosphate buffer pH 2.6. The chromatograms show that the retention time for 2-OH-E$^+$ is 12 min, and that the 2-OH-E$^+$ generated contains some contaminating ethidium (retention time 15 min).The retention time for dHEth was 20 min, and it can be seen that the dHEth contains some contaminating ethidium and 2-OH-E$^+$ which may be present as a consequence of oxidation of dHEth during storage and following exposure to light.*

As shown in the chromatograms (Fig. 2.15), the retention time of 2-OH-E^+ was found to be 12 min, and that of ethidium was shown to be 15 min. The 2-OH-E^+ synthesised exhibited a small peak at 15 min, suggesting the presence of contaminating ethidium, which was also reported by Zielonka et al. (2008). The retention time of dHEth was shown to be 20 min, and this standard contained ethidium and 2-OH-E^+ contamination, which occurs through storage and exposure to light, that can partially oxidise the probe (Zielonka et al. 2008).

It was next important to construct a standard curve of the 2-OH-E^+, Eth and dHEth standards, to enable the calculation of concentrations of the 2-OH-E^+ and the Eth products in the biological samples tested. Standards were prepared in a range of concentrations: 20 µM, 15 µM, 10 µM, 5 µM and 0 µM, and each concentration was run in triplicate and the HPLC analysis for the standard curves was performed three times (Fig. 2.16).

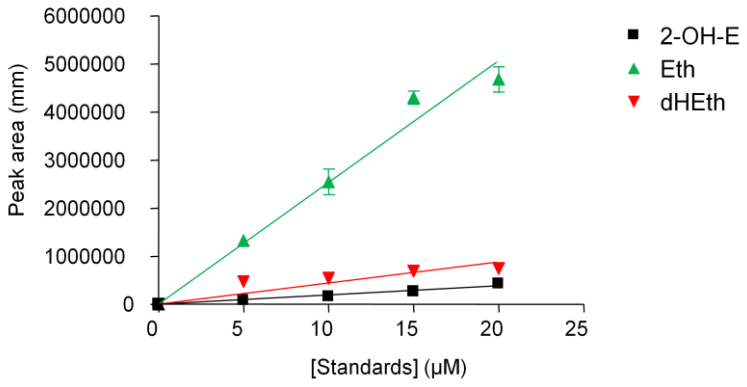

Figure 2.16. Standard curves of 2-OH-E^+, Ethidium, and dHEth. *Standard curves of the 2-OH-E^+, ethidium and dHEth standards were generated following dilution of the stocks to 5 µM, 10 µM, 15 µM and 20 µM. Each standard was run in triplicate and the HPLC was performed three times (n=3). The standard curve was then used to calculate the concentration of 2-OH-E^+ and ethidium present in the biological samples tested.*

2.2.4.4.2 Preparation of cells

Cells were prepared according to Zielonka et al. (2008). BV2 microglia were plated in 24 well plates at a density of $5x10^4$ cells per well and were grown to confluencey. The medium was changed to serum free medium 3 h prior to treatment as described in Table 1 for 24 h. After 24 h, cells were incubated with 5 µM dHEth for 40 min, after which the reaction was stopped by removing the culture medium and washing cells with ice-cold PBS. The cells were scraped immediately into 1 ml of ice-cold PBS and transferred to Eppendorf tubes on ice. The cells were then pelleted by centrifugation for 5 min at 1000 g at 4°C (Eppendorf 5415R benchtop centrifuge), and the supernatant was aspirated and the pellet was lysed in 150 µM ice-cold PBS containing 0.1% (v/v) of Triton X-100 using an insulin syringe. The un-lysed cells were spun down by centrifugation for 5 min at 1000 g at 4°C, and 100 µl of the supernatant was retained and added to fresh Eppenorf tubes containg 100 µM of 0.2 M $HClO_4$ in methanol, which promoted the precipitation of the protein from the lysates. The samples were left on ice for 2 h to allow protein precipitation. 2 µl of the remaining cell lysate was retained for the analysis of protien levels by Bradford assay (see section 2.2.6.2.2) which was used later to normalize the levels of 2-OH-E^+ and Eth to protein concentration. After 2 h, the precipitated protein was centrifuged for 30 min at 20,000 g at 4°C. 100 µM of the supernatant was transferred to Eppendorf tubes containing 100 µM phosphate buffer pH 2.6, and the excess buffer and $KClO_4$ was precipitated by centrifugation at 20,000 g at 4°C for 15 min. 150 µM of the supernatant was transferred HPLC vials which were placed into an autosampler cooled to 4°C (Jasco AS-2055 Plus). When all samples were prepared, the HPLC analysis was performed.

2.2.4.4.3 HPLC detection of 2-OH-E^+ and Eth

HPLC with fluorescence and UV-visible absorption detection was performed using a Jasco HPLC system equipped with UV (Jasco UV-975) and fluorescence (Jasco FP-1520) detectors. To separate 2-OH-E^+ and ethidium, 50 µM of each sample was injected into the HPLC system using a cooled autosampler (Jasco AS-2055 Plus) through a 1 cm x 4.6 mm Kromasil C_{18} guard column into a Kromasil C_{18} analytical column (3.9 x 150 mm, 5 µM particle size). A gradient elution method with two mobile phases was used to separate 2-OH-E^+ and ethidium: A – 50 mM phosphate buffer (pH 2.6), 10% acetonitrile, 90% water; and B – 50 mM phosphate buffer (pH 2.6) 60% acetonitrile buffer, 40% water. The elution conditions are shown in Table 4. The mobile phase was pumped through the column with a Jasco PU-1580 pump, with a flow rate of 0.4 ml / min which was used to pass the sample through the column to the detectors. With this set-up, the pressure in the column was maintained at 50-70 kg/cm^2, and each HPLC run took 60 min.

Time (min)	Mobile Phase A (%)	Mobile phase B (%)
0	80	20
10	55	45
30	45	55
40	0	100
46	0	100
50	80	20

Table 4. Elution conditions for the analysis of dHEth and 2-OH-E^+ and ethidium oxidation products by HPLC with fluorescence detection.

Fluoresence detection at 356 and 510 nm (excitiation) and 595 nm (emission) were used to detect 2-OH-E^+ and ethidium production, whilst absorbance at 220, 250, 290, 370 and 500 nm were used to monitor the levels of ethidium and dHEth not converted to either product in the sample. The sample was passed from the detector to the integrator which displayed the chromatogram in real time using Jasco EZChrom software. To quantify the dHEth derived

fluorescent products, the peak areas of 2-OH-E^+ and ethidium were measured and were compared with the standard curves generated from the 2-OH-E^+ and ethidium standards. Peaks at retention times of 12 min and 15 min were analysed, as these were shown to be the retention times of 2-OH-E^+ and ethidium respectively. No residual dHEth was observed in biological samples tested. Example chromatograms of control untreated cell lysates (Fig.2.17A) and of PMA cell lysates (Fig. 2.17B) are shown. The area / concentration correlation obtained from the 2-OH-E^+ and ethidium standards were used to calculate the concentrations of each product in the test sample by comparison. This was normalised for protein content and data was expressed as nmol (2-OH-E^+ or ethidium) / mg protein.

Initially, HPLC was performed on cell samples treated with the positive control PMA (10 ng/ml) and the NADPH oxidase inhibitor apocynin (10 µM) to ensure that the protocol was effective (Fig. 2.17C). As shown, the preliminary studies using PMA demonstrate that treatment of cells with PMA induces a significant increase in 2-OH-E^+ which could be significantly attenuated upon co-treatment of cells with apocynin, suggesting that PMA induces superoxide production in an NADPH oxidase dependent manner. Furthermore, control untreated cell and apocynin treated cells also only exhibit low levels of 2-OH-E^+ and Eth production (Fig. 2.17C). These investigations therefore suggested that HPLC analysis of dHEth oxidation products was suitable for the quantitative analysis of superoxide production in BV2 microglia.

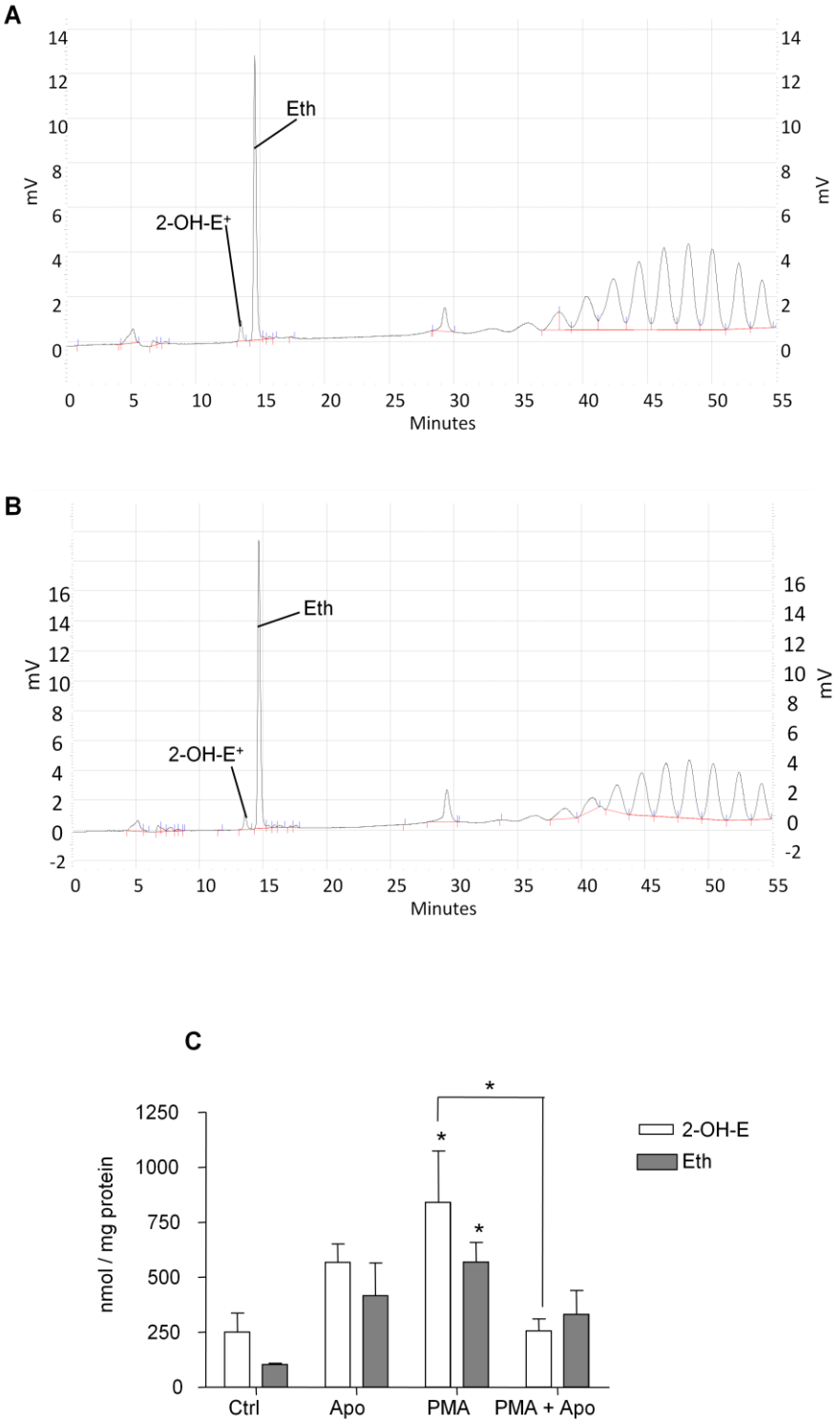

Figure 2.17 Example chromatograms and HPLC analysis dHEth oxidation products from cells treated with PMA in the presence or absence of apocynin. *The example chromatograms show peaks corresponding to 2-OH-E$^+$ (12 min retention time) amd ethidium (15 min retention time) in control* ***(A)*** *or PMA* ***(B)*** *treated BV2 microglia. Cells treated as shown in* ***(C)*** *were analysed in triplicate and the concentration of 2-OH-E$^+$ and ethidium were calculated from the peak areas which were compared with the known standards and data was normalized to protein concentration. Statistical analysis was performed using students T-Tests for comparison between treatments, and two way Anova for comparisons between 2-OH-E$^+$ and Eth for each treatment. *p<0.05. Data are n=3.*

2.2.5 NADPH oxidase activity assay

It is known that the NADPH oxidase converts molecular oxygen to superoxide through the oxidation of NADPH to NADP plus H^+, therefore an assay to determine the enzymatic activity of the NADPH oxidase was devised by Brightman et al. (1992). NADPH has an absorbance spectrum at 340 nm, and the reduction in absorbance at 340 nm is proportional to the decrease in NADPH through its consumption by the NADPH oxidase (Thannickal & Fanburg 1995). It was shown that NADPH has an absorption coefficient of 6.22 $mM^{-1}cm^{-1}$, which was used to calculate the amount of NADPH consumed during the assay (Thannickal & Fanburg 1995). For investigation into specific oxidase activities, for example the NADPH oxidase, Thannickal & Fanburg (1995) used DPI, an NADPH oxidase inhibitor to show that activation with a specific stimuli induced NADPH oxidase activation. Here, the same approach has been used with apocynin to show that the NADPH oxidase could be activated with the stimuli shown in Table 1.

2.2.5.1 Optimised protocol for NADPH oxidase activity assay

The protocol followed was optimised from Carmona-Cuenca et al. (2008). BV2 microglia were plated in 6 well plates at a density of $1x10^5$ cells per well and were grown to confluency. After this time, medium was changed to serum free media for 3 h before cells were activated initially with the known NADPH oxidase activators PMA (10 ng/ml) and LPS (1 μg/ml). Cells were treated in the presence or absence of apocynin, administered 30 min before the reading (Thannickal & Fanburg 1995) so that NADPH reduction could be attributed to activity of the NADPH oxidase rather than any other system. To determine the optimum time point at which the NADPH oxidase was significantly active when compared to control untreated cells or apocynin treated cells, a time course analysis was performed (Fig. 2.18) which showed that 4 h incubation with the activators PMA (Fig. 2.18A) or LPS (Fig. 2.18B) induced a significant increase in NADPH oxidase activity, which could be inhibited by

apocynin. Therefore during the assay, cells were incubated at 37ºC at 6% CO_2 for 4 h with the activators shown in Table 1. After this, cells were removed from the incubator, and were washed once with PBS, before detachment from the culture vessels using 500 µl accutase. Once detached, accutase was neutralised with an equal volume of serum containing media. The 1 ml cell suspension was transferred to an Eppendorf tube and centrifuged at 2500 g at 4ºC (Eppendorf 5415R benchtop centrifuge) for 5 min. The resulting pellet was resuspended in 300 µl PBS and transferred to a 96 well plate, where 100 µl of each sample was transferred to each well (therefore each sample was tested in triplicate). Immediately before the plate was read in the plate reader, 250 µM NADPH was added to each well, and the decrease in absorbance as NADPH was converted to NADP was measured at 340 nm every minute for 10 min using a Tecan x fluo4 plate reader. The raw data were plotted and a graph showing the gradient was used to calculate the initial rate of activity.

To determine the protein concentration in each sample, an aliquot of cells were lysed in lysis buffer and a Bradford protein assay was performed on each sample (see section 2.2.6.2.2). This information, along with that of the initial rate of activity and the absorbance extinction co-efficient for NADPH ($6.22 mM^{-1}cm^{-1}$) then allowed determination of how many micromoles one microgram of protein could convert to product in 1 min. Data was expressed in µM NADP / µg protein / min, and was calculated using the equation C=A/(ExL) where C is the concentration of NADP reduced by the NADPH oxidase, A is the absorbance (the gradient of the initial rate of reaction), E is the extinction coefficient of NADPH ($6.11 mM^{1}cm^{-1}$) and L is the path length (1 cm).

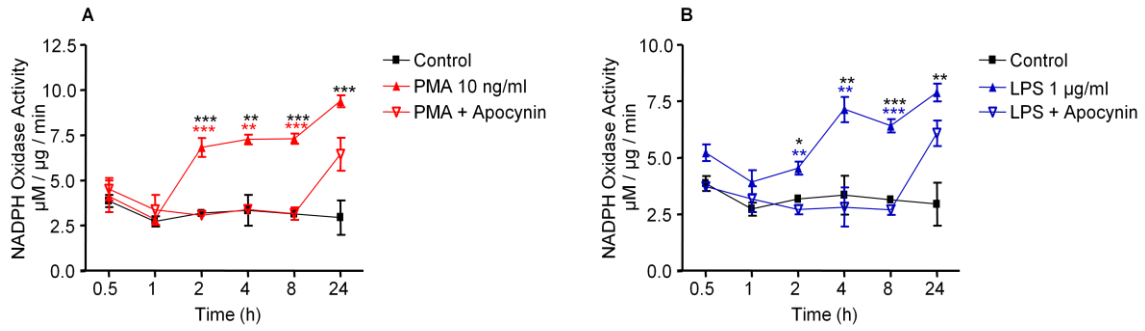

Figure 2.18 Time course analysis of NADPH oxidase activity. *BV2 cells were treated with the NADPH oxidase activators PMA 10 ng/ml (**A**) or LPS 1 µg/ml (**B**) in the presence or absence of apocynin 10 µM administered for 30 min before the reading. The time-course analysis shows that there is a significant increase in NADPH oxidase activity, which can be inhibited by apocynin at 2, 4, and 8 h incubation. Statistical analysis was performed using a one way ANOVA with Tukey post-hoc analysis, and comparisons were made between treatment and treatment plus apocynin and treatment and control groups. ***p<0.001, **p<0.01 and *p<0.0. All data are n=3.*

2.2.6 Expression analysis

2.2.6.1 mRNA analysis using reverse transcription and polymerase chain reaction

Reverse transcription and polymerase chain reaction (RT-PCR) enables the analysis of expressed (transcribed) genes of interest. The method is semi-quantitative, however provides an insight into modulation of gene expression. The protocol has four stages: RNA extraction, in which total RNA is isolated from the cell culture; reverse transcription, in which a complementary (cDNA) copy of the RNA is produced from the cells using an RNA dependent DNA polymerase; PCR in which the gene of interest is amplified using specific primers for sections of the gene to be analysed; and finally the PCR products are resolved by agarose gel electrophoresis and imaged using a UV-transilluminator.

The principle behind RT-PCR therefore depends on the extraction of RNA which can be converted into cDNA, and also on the specificity of the PCR primers. PCR primers are designed complementary to a section of the cDNA of interest, therefore ensuring that the PCR reaction is specific to a particular gene.

2.2.6.1.1 RNA extraction

Primary microglia were used for RT-PCR analysis. Cells were plated at $1x10^6$ cells per well in 6 well plates, and following serum deprivation for 3 h, cells were treated according to Table 1 for 24 h before RNA was extracted. RNA extraction was performed using a modified acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi 1987). Cells were lysed with 1 ml Trizol and incubated at room temperature for 5 min, before 200 µl chloroform was added and samples were shaken vigorously then incubated at room temperature for 2 min to begin the separation of protein, DNA and RNA. Samples were centrifuged at 11600 g for 15 min at 4 ºC (Eppendorf 5415R benchtop centrifuge). Chloroform and centrifugation promotes phase separation, in which RNA can be found in the top aqueous layer, DNA is found in the white middle layer, and protein is isolated in the pink lower layer. The top aqueous layer was therefore removed and retained, and from this, RNA was precipitated with 500 µl isopropyl alcohol, followed by incubation at room temperature for 10 min. The samples were then centrifuged again at 11600 g for 10 min at 4 ºC, and the supernatant was discarded leaving the RNA pellet, which was washed with 1 ml 75% ethanol and centrifuged at 6200 g for 5 min at 4 ºC. The supernatants were discarded and the pellet was air dried then dissolved in an appropriate volume of DEPC dH_2O (typically 10 µl). Samples were heated to 60 ºC for 10 min to aid dissolving. RNA concentration was measured by absorbance at $A_{260/280}$ (Ultrospec 2000 UV/Visible spectrophotometer, Pharmacia Biotec). The $A_{260/280}$ ratio indicates the purity of the RNA, where pure RNA has a ratio of 2. RNA concentration was calculated using the Beer-Lambert law, which states that:

A_{260} = RNA extinction coefficient (25 $\mu l.\mu g^{-1}.cm^{-1}$ x RNA concentration ($\mu g.\mu l^{-1}$)) x path length (1 cm).

Therefore, RNA concentration ($\mu g.\mu l^{-1}$) = A_{260} x 40 x dilution factor

In this case, [RNA] ($\mu g.\mu l^{-1}$) = A_{260} x ((400/3) x 40 / 1000) = A_{260} x (16/3)

The RNA concentration was required to determine the volume of isolated RNA required to reverse transcribe 3 µg RNA in the next step of the reaction.

2.2.6.1.2 Reverse transcription

Total RNA was reverse transcribed to cDNA using the Superscript Reverse Transcriptase kit (Invitrogen) according to manufacturer's instructions. The superscript reverse transcriptase is isolated from the Moloney murine leukaemia virus (M-MLV) and is an RNA dependant DNA polymerase used to synthesise first strand cDNA for use in PCR. For each reaction 3 µg RNA was used, which was first incubated with 1 µL Oligo dT primer$_{12\text{-}18}$, 1 µl 10 mM dNTP mix and distilled water to 12 µl. The solution was incubated at 65 ºC for 5 min, to promote annealing of the primer to the single stranded RNA. The reaction was then quenched on ice. Following this, 4 µl 5 x first strand buffer, 2 µl 0.1 M DTT and 1 µl 40 U/ml ribonuclease inhibitor RNase out was added to each sample, resulting in a final volume of 19 µl. The reaction was then incubated at 42 ºC for 2 min to prime the reaction before the addition of 1 µl (equivalent 200 U / reaction) of Superscript II Reverse Transcriptase, giving a final volume of 20 µl. Samples were then incubated at 42 ºC for 50 min, and the reaction was inactivated by heating to 70 ºC for 15 min. The resulting cDNA samples were subjected to PCR analysis. A sample was run alongside each RT reaction that contained no reverse transcriptase, as a negative control to check for contamination with DNA, which could occur during the RNA extraction stage. This was subjected to PCR with primers for a housekeeping gene such as β-actin.

2.2.6.1.3 Polymerase Chain Reaction (PCR)

PCR amplification of reverse transcribed RNA results in the production of a single amplicon, as only the gene of interest is transcribed due to the use of unique primers to amplify a small region of the cDNA of interest. PCR amplification of a gene of interest relies on temperature changes that direct the annealing of primers, the elongation of the sequence from the primer sites and finally the melting of the double stranded product to enable further annealing of primers, and subsequent elongation (Fig. 2.19).

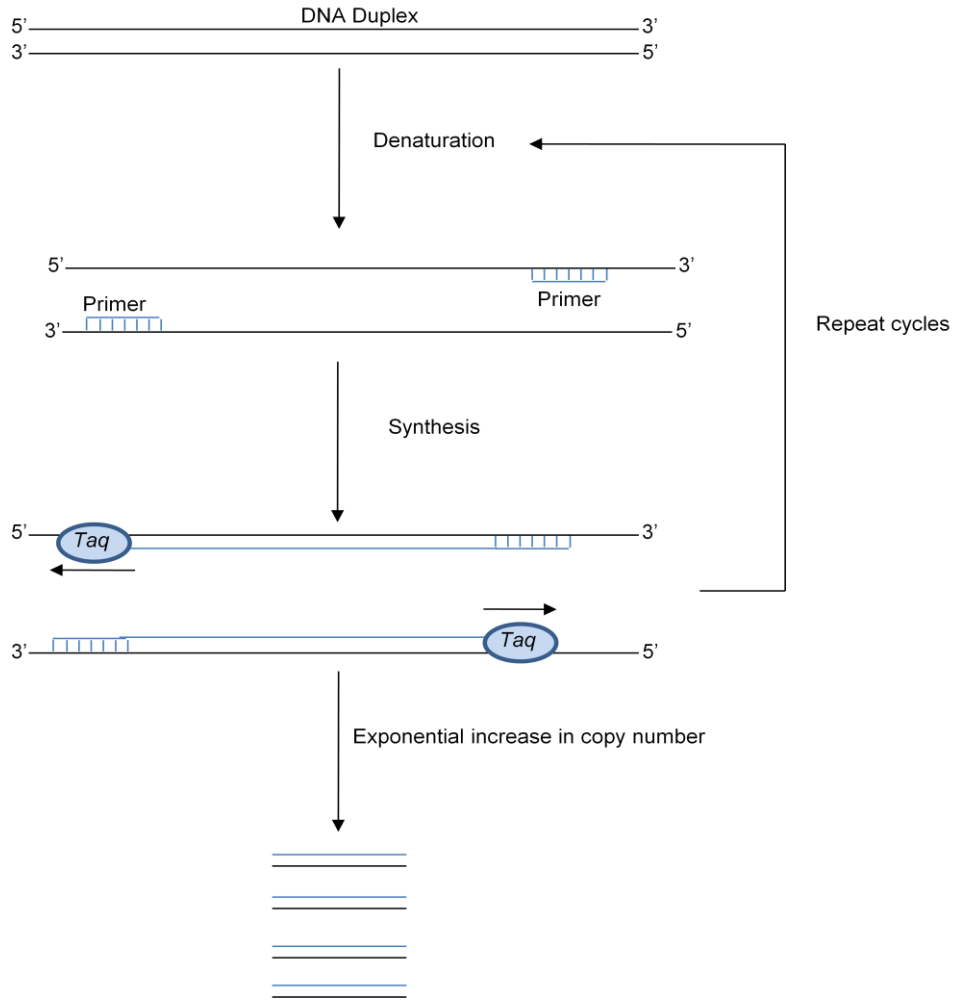

Figure 2.19 The polymerase chain reaction. *PCR begins with the melting of the DNA duplex using high temperatures to break the bonds between the base-pairs. Primers then anneal to their complementary DNA, and Taq polymerase synthesizes the complementary strand by aiding the addition of dNTP's to the primer. This is repeated, and gives rise to an exponential increase in copy number of the DNA.*

The process is exponential, producing a high copy number of DNA. Temperature changes therefore control PCR, with an initial high temperature (typically in the region of 95ºC) driving the melting of double stranded DNA to expose the complementary sites for the annealing of the primers. The next temperature therefore determines the annealing of the primers, and is termed the optimal annealing temperature (OAT), which is calculated for each individual primer and is averaged for the primer pair. This is calculated by:

$OAT = T_m - 4ºC$

Where T_m is the melting temperature of the DNA, which is calculated from the G/C content, and is calculated by:

$T_m = 4(G+C) + 2(T+A)\ ºC$

The interaction between G-C base pairs involves 3 hydrogen bonds, which requires a higher energy to break than the A-T base pairs, which only contains 2 hydrogen bonds. The melting temperature is therefore driven by G-C content, and primers are designed to contain approximately 50% G/C bases, as this provides a strong bond between the template DNA and primer, without requiring too high an annealing temperature. The annealing temperature is typically around 60ºC which must be significantly different from the elongation temperature. In the elongation phase, the DNA polymerase, *Thermophilius Aquqticus (Taq)* is used to polymerise the formation of a complementary strand of DNA from the primers using the dNTPs in the PCR mix. The *Taq* polymerase was isolated from the *Thermophilius Aquaticus* bacterium found in hot springs, which enables it to withstand the high temperatures of PCR. Typically the elongation stage is carried out at 72ºC, the optimum temperature for *Taq* polymerase activity. Each dissociation- annealing – elongation cycle doubles the amount of DNA so that the reaction increases DNA product exponentially. With each gene of interest

analysed in this thesis, the number of cycles was optimised to ensure that no artefacts of the PCR reaction were generated.

Here, cDNA was subjected to PCR amplification to investigate the expression of Nox1, Nox2, Nox4, TNFα, and the GABA receptor subunits, using β-actin as a housekeeping gene in microglia and CGCs (primer sequences shown in Table 4). The Promega GoTaq Flexi DNA polymerase kit was used to amplify DNA according to manufacturer's instructions. Each reaction contained 2 µl cDNA (reverse transcribed from total RNA as above), and 10 µl 5X Go-Taq flexi buffer, 2.5 mM MgCl (2 µl), 1 µl of 10 mM dNTPs, 2.5 µM each primer (primers were custom made by Sigma and were supplied at 100 µM, sequences shown in Table 5), 5 U/ml GoTaq polymerase (0.25 µl) and nuclease free water to 50 µl.

PCR was performed in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems) using the following conditions: 95ºC for 2 min for initial annealing of primers, followed by 30 cycles of 95ºC for 45 s for separation of double stranded DNA, 59ºC for 45 s for primer annealing, 72ºC for 45 s for extension and elongation of the DNA construct, followed by a final elongation stage at 72ºC for 10 min, and finally 4ºC for storage.

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')	Fragment Size
Nox1 Rat	CATAAGATGGTAGCTTGGAT	GAGTGCTACTGAATATGGGT	115 bp
Nox2 Rat	AAGCCATTGGATCACAACCT	GGCTTCTACTGTAGCGTTCA	123 bp
Nox4 Rat	TGACTGAGGTACAGTGGATGTT	CCTGAGGTTACAGCTTCTACCT	100 bp
TNFα	CCCAGACCTCACACTAGTATC	TCGTAGCAAACCACCAAGCAG	150 bp
$GABA_A$ Receptor α1 subunit	TGACAGTCATTCTCTCCCAAGTC	TCAGAACGGTCGTCATCC	100 bp
$GABA_A$ Receptor α2 subunit	GGTTTCCGCTGCTTGTTCT	TTCTTGGATGTTAGCCAGCAC	150 bp
$GABA_A$ Receptor α3 subunit	GGTCATGTTGTTGGGACAGA	TCAGATGAAAGTGGGTTGTCA	173 bp
$GABA_A$ Receptor α4 subunit	CTTCTGGATCAATAAGGAGTCTG	TCATCGTGAGGACTGTGGTT	125 bp
$GABA_A$ Receptor α5 subunit	GGTCTTGGATGGCTATGAC	ACCTGCGTGATTCGCTCT	110 bp
$GABA_A$ Receptor α6 subunit	AATGTCAGTCGGATTCTTGACA	TTTTGACCTCTGTTACAGCACCT	150 bp
$GABA_A$ Receptor β1 subunit	CAAACTGGAGATGAACAAAGTC	CCAGGGTGCTGAGGAGAATA	125 bp
$GABA_A$ Receptor β2 subunit	GGGTCTCCTTTTGGATCAACTA	GGTCATCGTCAGGACAGTTGTA	683 bp
$GABA_A$ Receptor β3 subunit	TCATGGGTGTCCTTCTGGAT	ATGGTGAGCACGGTAAT	500 bp
$GABA_A$ Receptor γ1 subunit	TCATGGACTATGGAACCTTGC	GCATGGAGGACCTGGGGTA	175 bp
$GABA_A$ Receptor γ2 subunit	TGCTCACTGGATCADGACTC	ATTGCAACTGGCACTCAGC	150 bp
$GABA_A$ Receptor γ3 subunit	AGCCTTCAAGCACCCTCTAA	CGTGCCAGTACATGGAATTG	150 bp
β-actin Mouse, Rat	ATCGTCGGCCGCCCTAGGCAC	TGGCCTTAGGGTTCAGAGGGGC	243 bp

Table 5. PCR primers *Primers were designed using the Roche primer design tool and generated by Sigma. β-actin primers were from sigma, and are standard non-custom primers.*

2.2.6.1.4 Resolution of PCR products

The PCR products were resolved on a 1% agarose gel (agarose dissolved in 1x Tris/borate/EDTA (TBE) buffer), containing 0.5 μg/ml ethidium bromide, and were visualised by UV illumination of intercalated ethidium bromide using a UV transilluminator (GelPro Imager). Images were captured using a CoolSNAP-Pro camera, and band densities were determined using ImageJ. The densities of the bands correspond directly to the amount of mRNA in the original sample.

2.2.6.2 Western blotting

Western blotting is a gel electrophoresis and blotting method used to separate and identify proteins of interest in a biological sample. The technique was used in this thesis to determine the expression levels of different proteins of interest. Here, denaturing gels were used, and the Western blotting protocol relied upon the denaturation of all expressed proteins in the cell, of which single proteins could be identified by immunoblotting with antibodies directed against epitopes of the proteins of interest. The technique begins with cell lysates, which are denatured by the addition of sodium dodecyl sulphate (SDS) which cleaves disulphide bonds, reverting the proteins to their primary structure. SDS also coats the proteins in a negative charge, so that they may not re-fold. Following lysis, lysates are subjected to a protein assay to determine the amount of total protein present, so that an equal amount may be loaded onto the gel to ensure that any differences in band intensity are a result of modulation of protein expression rather than a difference in loading. Following the loading of protein into a sodium dodecyl sulphate polyacrylamide gel, electrophoresis is performed (SDS - PAGE) by passing an electrical current through the gel so that the negatively charged proteins migrate to the positive electrode (the cathode). Polyacrylamide gels are made with different percentages of acrylamide according to the molecular weight of the protein of interest, as lower molecular weight proteins migrate faster through the gel than higher molecular weight proteins. To

investigate a low molecular weight protein, a higher percentage gel is required. Following SDS-PAGE, proteins are transferred onto a polyvinylidine fluoride (PVDF) membrane, which is a positively charged material that the negatively charged proteins bind to. Following transfer, the membrane is incubated with primary antibodies of interest which are raised against a specific epitope of a protein of interest. To visualise the bands on the membranes, secondary antibodies conjugated to horseradish peroxidise (HRP) are used, and visualisation is by enhanced chemiluminescense (ECL).

2.2.6.2.1 Cell lysis and sample preparation

CGCs, and primary microglia were used for Western blot analysis. Cells were prepared as described previously and were lysed in 15 µl lysis buffer containing 20 mM Tris-acetate buffer (pH 7), 1 mM ethylene diamine tetraacetic acid (EDTA) to chelate calcium, 1 mM ethylene glycol tetraacetic acid (EGTA) to chelate magnesium, 10 mM of the phosphatase inhibitor sodium-β-glycerophosphate, 1 mM of the tyrosine phosphatase sodium orthovanadate, 5% glycerol to depress the solution's freezing temperature, 1% Triton X-100 membrane solubiliser, 0.27 M sucrose, 1 mM of the protease inhibitor benzamidine, 4 µg/ml of the protease inhibitor leupeptin, 1 µM of the phosphatase inhibitor microcystin LR and 0.1% of the reducing agent β-mercaptoethanol (Evans & Pocock, 1999; Kingham & Pocock, 2000). Samples were left on ice for 30 min and then centrifuged for 5 min at 10 000 g to pellet the nuclear fraction. Supernatants (the cyosolic fraction) were transferred to new eppendorf tubes and all samples were stored at -20^{o}C until used. The formulation of the lysis buffer is such that the buffer lyses the cells and also solubilises the membranes, in addition to preserving the proteins at the time of lysis by preventing phosphorylation, dephosphorylation and proteolysis.

2.2.6.2.2 Bradford assay

The Bradford assay was used to determine the total protein concentration of the sample (Bradford, 1979). As all proteins of interest studied in this thesis were cytosolic, the cytosolic fractions were measured for protein content. In a 96 well plate, 1 µl of each sample was placed into one well – each sample was measured in triplicate, and 200 µl Bradford reagent was added onto this. Samples were shaken and left at room temperature for 5 min before the absorbance shift of the Coomassie dye towards the blue spectrum following the binding of protein was measured at 595 nm on a Tecan X Fluor 4 plate reader. The absorbance of each sample was compared to a standard curve of BSA of known concentrations, constructed on the same plate as each experimental sample. From this, the volume of lysate required for each sample to contain 45 µg of protein was derived, and samples were removed to another Eppendorf tube. To these samples, an equal volume of sample buffer (2% SDS, 10% glycerol, 2.5% mercaptoethanol, 125 mM Tris/HCl (pH 6.8) and a few bromophenol blue crystals (Laemmli, 1970)) was added to denature the protein, and to aid loading and visualisation of the gel front. Samples were then boiled for 5 min before loading.

2.2.6.2.3 SDS-PAGE

Bio-Rad mini gel apparatus were used to cast polyacrylamide gels fresh on the day of the experiment. As mentioned previously, the percent of the gel was changed depending on the molecular weight of the protein of interest. Typically, gels were used at 10% and 12% polyacrylamide (Table 5), with the higher percentage gel used for the separation of lower molecular weight proteins. Gels were cast between two glass plates separated by spacers mounted on a pouring stand. Resolver gel was made first and poured between the glass plates (Table 6). The resolver buffer was composed of 1.4 M Tris Base and 0.1% SDS at pH 8.8. The ammonium persulphate (APS) and the N, N, N', N'-tetramethylethylenediamine

(TEMED) are setting agents added to the gel just before pouring. Gels were topped wth 100% ethanol to level off the interface and were left to set for around 1 h.

Resolver Gel 10%	**Resolver Gel 12 %**	**Stacker gel 4%**
3.33 ml 30% Polyacrylamide	4 ml 30% Polyacrylamide	1.33 ml 30% polyacrylamide
2.5 ml Resolver buffer (pH 7.8)	2.5 ml Resolver buffer (pH 7.8)	2.5 ml Stacker buffer (pH 6.8)
4.17 ml H_2O	3.5 ml H_2O	6.17 ml H_2O
50 µl 10% APS (fresh)	50 µl 10% APS (fresh)	50 µl 10 % APS (fresh)
5 µl TMED	5 µl TMED	5 µl TMED

Table 6. Composition of resolver gels for 10% and 12% gels and stacker gel

Stacker gels were layered on top of the set resolver gels. The stacker gels were made as shown in Table 5 with stacker buffer made of 0.5 M Tris base and 0.1% SDS at pH 6.8. A comb was placed into the gel, which was left to set for a further hour.

Once set, the gels were placed into a holder and into a gel tank, which was filled with running buffer (125 mM Tris/HCl, 1 M glycine, 0.01% SDS) and the prepared samples were loaded into each well. Gels were electrophoressed at 150 V until the proteins were separated completely. After the proteins had separated, the gels were removed from the tanks and equilibrated in ice cold transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.01% SDS) for 20 min. At the same time, fiber pads and 3 mm filter paper was also soaked in transfer buffer. Whilst the gel was equilibrating, the PVDF membrane was activated in ice cold methanol for 1 min, after which the membrane was washed in dH_2O for 5 min. The membrane was then soaked in transfer buffer until the gels were ready for transfer.

2.2.6.2.4 Transfer

Proteins were transferred onto PVDF membrane using a wet transfer system. One pre-soaked fibre pad was placed onto the black side of the transfer cassette. A piece of 3 mm filter paper was put on top of this, and then the gel was laid onto the paper. The membrane was then carefully laid over the gel, and a further piece of 3 mm filer paper was put over that. Air

bubbles were removed from the stack, and the final fibre pad was added, and cassettes were then closed. The cassettes were then placed into a holder which was put into a tank filled with transfer buffer and an ice pack. A current of 80 V was applied for 1 h which allowed proteins to transfer onto the membrane. The proteins migrated from the negative anode to the positive cathode, and attached to the positively charged membrane. Following transfer, membranes were washed in Tween-20 Tris buffer saline (TTBS: 10 mM Tris HCl, 150 mM NaCl, 0.5% Tween-20, pH 7.4) for 10 min, and membranes were then blocked in 5% non-fat milk solution in TTBS for 1 h to prevent non-specific binding of the antibody. The membranes were then ready for immunoblotting.

2.2.6.2.5 Immunoblotting

After blocking with 5% non-fat milk solution, membranes were incubated with the primary antibody diluted in 5% non-fat milk as shown in Table 7. Typically membranes were incubated with the primary antibodies overnight at 4ºC on an orbital shaker.

Primary Antibody	Dilution and incubation	Corresponding secondary antibody	Dilution and incubation
Rabbit anti mouse iNOS (BD transduction lab 610333)	1:5000 2 h room temp	Goat anti-rabbit IgG peroxidise (Sigma A-0545)	1:2000 1 h room temp
Rabbit monoclonal anti phospho-p38MAPK (Thr 180/ Tyr 182) (Cell Signalling Technology 9211S)	1:1000 overnight 4°C	Goat anti-rabbit IgG peroxidise (Sigma A-0545)	1:2000 1 h room temp
Rabbit polyclonal anti total p38MAPK (Santa Cruz SC-7149)	1:1000 overnight 4 °C	Goat anti-rabbit IgG peroxidise (Sigma A-0545)	1:2000 1 h room temp
Rabbit monoclonal anti phospho-p44/42MAPK (Thr 202 / Tyr 204) (Cell Signalling Technology 9100S)	1:1000 overnight 4°C	Goat anti-rabbit IgG peroxidise (Sigma A-0545)	1:2000 1 h room temp
Rabbit monoclonal anti phospho-Akt (Ser 473) (Cell Signalling Technology 4060B)	1:1000 overnight 4°C	Goat anti-rabbit IgG peroxidise (Sigma A-0545)	1:2000 1 h room temp
Rabbit polyclonal anti Caspase 12 (full length and cleaved) (Abcam ab18766)	1:1000 overnight 4°C	Goat anti-rabbit IgG peroxidise (Sigma A-0545)	1:2000 1 h room temp
Rabbit polyclonal anti-TNFR1 (Santa Cruz SC-7895)	1:1000 overnight 4°C	Goat anti-rabbit IgG peroxidise (Sigma A-0545)	1:2000 1 h room temp
Goat polyclonal anti-TNFα (Santa Cruz SC-32155)	1:1000 overnight 4°C	Bovine anti-goat IgG HRP (Santa Cruz SC-2384)	1:2000 1 h room temp
Mouse anti rabbit beta actin (Sigma A2066)	1:2000 2 h room temp	Rabbit anti-mouse IgG HRP (Santa Cruz SC – 358914)	1:1000 1 h room temp

Table 7. List of primary and secondary antibodies used for Western blotting.

Following incubation with the primary antibodies (Table 7), the membranes were washed three times with TTBS and then incubated with the secondary antibodies as shown in Table 6 for 1 h at room temperature on an orbital shaker. The membranes were then washed a further three times with TTBS and were exposed using enhanced chemiluminescence (ECL). Membranes were drained and placed in a tray containing an equal volume of each ECL solution and were incubated for 1 min. Membranes were then blotted on filter paper to remove excess ECL solution, and were wrapped in saran wrap and fixed in an X-ray cassette. In the dark, a piece of photographic film was placed onto the membranes to expose the protein bands. Photographic paper was then developed in developing solution and immersed in fixing solution before finally being rinsed in water. The films were dried and analysed and the expression of each protein was quantified using densitometry (ImageJ). Western blots were performed three times and those presented here are representative blots. Blots were routinely re-probed for β-actin as a loading control to ensure equal loading of protein, which was also used for densitometry.

2.2.7 Enzyme linked immunosorbent assays (ELISA) of TNFα release

Enzyme-linked immunosorbent assays (ELISAs) quantify the amount of protein in a sample (Engvall & Perlmann, 1971; Van Weemen & Schuurs, 1971). A quantitative sandwich ELISA kit was used here to measure the quantity of TNFα released in control and treated cell culture medium samples from microglia treated as shown in Table 1. In the sandwich ELISA a primary monoclonal antibody is bound to the surface of a well in 96 well plate format, which enables the antigen under investigation to bind directly onto the bound antibody (Belanger et al. 1973). The antigen is then visualised by a second polyclonal antibody that binds to it, and the signal is amplified and visualized using a plate reader.

Microglia were treated as outlined in Table 1, and media was removed and centrifuged at 10 000 g for 5 min to pellet debris. The resulting supernatant was placed into fresh Eppendorf tubes and samples were frozen until required. When the assay was perfomed, samples were defrosted on ice. The assay was performed according to the manufacturer's instructions, in 96 well plates precoated with a monoclonal rat TNFα specific antibody. First, 50 µl of assay diluent were added to each well, followed by 50 µl of standard rat TNFα samples of known concentrations, TNFα kit control or media samples. The samples were incubated for 2 h at room temperature followed by four washes with wash buffer. Samples were then incubated with 100 µl of rat TNFα conjugate for 2 h at room temperature, which was followed by four washes with wash buffer. 100 µl of substrate solution was added to the wells for 30 minutes in the dark, after which, 100 µl of stop solution was added to terminate the reaction. The wells were mixed and optical density was measured with a microplate reader at 450 nm with a reference filter at 540 nm to correct for optical imperfections. The concentration of TNFα in the cell medium samples was determined against a standard curve from the standard TNFα samples used on the same 96 well plate.

2.2.8 Statistical analysis

To assess statistical significance in all experiments, experiments were performed in triplicate using three separate cell preparations. In imaging experiments, three coverslips were imaged per condition, and a minimum of six fields of view per coverslip were imaged and analysed. Where percentages were calculated, statistical analysis was performed on transformed data. Statistical analysis was performed using the GraphPad Prism software. Data were analysed using one-way ANOVA with Tukey post-hoc analysis, a two-way ANOVA (for HPLC analysis), or the Student's T test. Significance was stated as $*p<0.05$, $**p<0.01$, $***p<0.001$.

Chapter 3

Neurotransmitter modulation of superoxide production in microglia

3.1 Introduction and summary of results

Neurotransmitter dysregulation is a hallmark of several neurodegenerative conditions (Greenfield & Vaux 2002), and is a consequence of neuronal damage induced by insults such as Aβ plaques, α-synuclein deposits, traumatic injury, and ischemia, resulting in neurotransmitter release into the extracellular matrix (Vesce et al. 2007; Rossi & Volterra 2009). Microglia have the capacity for neurotransmitter uptake (Rimaniol et al. 2000), and express a wide repertoire of neurotransmitter receptors (Pocock & Kettenmann 2007), which enables them to respond to both physiological and pathophysiological levels of neurotransmitters in the extracellular space. Furthermore, ROS production is a hallmark of almost all neurodegenerative conditions (Patel & Chu 2011), and can be either protective (Groeger et al. 2009), or can enhance disease progression (Abramov et al. 2007). Neurotransmitter dysregulation and ROS production are therefore key events in the progression of neurodegenerative disease.

Glutamate is implicated in almost all neurodegenerative conditions, with excessive release seen during stroke, AD, (Rossi & Volterra 2009) and HD (DiFiglia 1990); whilst GABA tone is increased in AD pathology (Marczynski 1998), and ATP is released from degenerating neurons to recruit microglia to sites of neuronal injury (North & Verkhratsky 2006). Modulation of microglial neurotransmitter receptors affects microglial reactivity (Pocock & Kettenmann 2007), and as ROS are a hallmark of microglial activation (Block 2008) it could be suggested that modulation of microglial neurotransmitter receptors may mediate microglial activity through activation of the NADPH oxidase, which was therefore investigated here.

As neurotransmitter release in the CNS is a key pathological event, and microglial ROS production has pathological or protective consequences, the aim of this chapter was to

determine whether neurotransmitters and modulation of neurotransmitter receptors could influence microglial superoxide production through activation of the NADPH oxidase.

Treatment of microglia with the neurotransmitters glutamate, GABA or BzATP induced superoxide production in BV2 and primary microglia. Furthermore, superoxide production was a consequence of NADPH oxidase activation, as neurotransmitter induced superoxide production was inhibited by co-treatment of cells with the NADPH oxidase inhibitor apocynin.

Modulation of the microglial mGluRs induced superoxide production in an NADPH oxidase dependent manner. Inhibition of the group I mGluRs with 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride (MTEP), activation of the group II mGluR3 with *N*-Acetyl-L-aspartyl-L-glutamic acid (NAAG), and activation of the group III mGluRs with L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4) significantly elevated NADPH oxidase derived superoxide production. In addition, activation of the NMDA receptor, but not the AMPA receptor promoted superoxide production in an NADPH oxidase dependent manner. Furthermore, NMDA derived superoxide was attenuated by co-treatment with the receptor agonist and antagonist, lending support to findings that microglia express functional NMDA receptors (Murugan et al. 2011).

Microglia also responded to $GABA_A$ receptor activation, and treatment with muscimol significantly elevated superoxide production as a consequence of NADPH oxidase activation. Furthermore, this was a consequence of receptor activation, as co-treatment of microglia with the $GABA_A$ receptor agonist and the highly specific $GABA_A$ receptor antagonists 6-Imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid hydrobromide (SR95531) or picrotoxin attenuated superoxide production. Microglia were also shown to express subunits of the $GABA_A$ receptor required for the formation of functional receptors, in support of findings

from Lee et al (2011). Finally, activation of the microglial P2Y2/4 receptor also induced superoxide production in an NADPH oxidase dependent manner.

In this chapter, four methods were employed to perform a thorough investigation into microglial superoxide production. The NBT assay was used to detect superoxide production in BV2 microglia, as this method is highly sensitive to superoxide specifically. To validate the NBT findings, the dHEth probe was used. Superoxide production in primary microglia was assessed by dHEth fluorescence microscopy, and was quantified by flow cytometry and HPLC, in which the specific oxidation products of dHEth were detected to determine whether superoxide or H_2O_2 were produced following neurotransmitter and receptor induced NADPH oxidase activation. To attribute superoxide production to the NADPH oxidase, all treatments were performed in the presence or absence of the NADPH oxidase inhibitor apocynin, whereby attenuation of superoxide production upon co-treatment with apocynin suggested that the treatments induced superoxide production in an NADPH oxidase dependent manner. To further implicate the NADPH oxidase in superoxide production following modulation of neurotransmitter receptors, enzymatic activity was analysed using the NADPH oxidase activity assay.

It was anticipated that this robust analysis into superoxide production and NADPH oxidase activity would provide strong evidence to show that modulation of the mGluRs, iGluRs, GABA and purinergic receptors mediate microglial superoxide production in an NADPH oxidase dependent manner.

3.2 Results

3.2.1 Glutamate, GABA and ATP induce superoxide production in microglia which is due to NADPH oxidase activation

Initially, it was important to determine whether neurotransmitters could induce superoxide production in microglia through activation of the NADPH oxidase, before further investigations into neurotransmitter receptor mediated superoxide production were performed.

The NBT assay was used first to determine whether neurotransmitters induced superoxide production in BV2 microglia, and to determine the optimal concentration of glutamate, GABA or BzATP required to promote superoxide production. Initially a concentration titration of the neurotransmitters glutamate (Fig. 3.1A), GABA (Fig. 3.1B), and BzATP (Fig. 3.1C) was performed. The NBT analysis revealed that treatment of BV2 microglia with glutamate at 1 µM significantly increased NBT reduction following incubation of cells for 4 h. Treatment of BV2 microglia with 1 µM or 100 µM GABA induced a significant increase in NBT reduction (Fig. 3.1B), however, as treatment with 100 µM GABA promoted higher levels of superoxide production (**$p<0.01$), this concentration was used for all further experiments. Treatment of BV2 microglia with 250 µM or 1 mM BzATP significantly increased NBT reduction (Fig. 3.1C). BzATP was used in place of ATP, as ATP was rapidly hydrolysed in the culture media, and could not exert an effect *in vitro* (Parvathenani et al. 2003). The findings that the highest concentration of 1 mM BzATP induced a significant increase in superoxide production is in line with published data showing that this concentration induces microglial reactivity through the opening of a large pore in the membrane, which enables the passage of large (900 Da) molecules across the membrane, and also promotes microglial phagocytosis and chemotaxis (Skaper et al. 2010) . This was not

under investigation in this thesis, therefore the lower concentration of 250 µM was used, in agreement with Parvathenani et al. (2003).

A time course NBT assay was then performed following the findings that 1 µM glutamate, 100 µM GABA and 250 µM BzATP were optimal concentrations for the production of superoxide in BV2 microglia. The time course analysis was carried out to confirm that 4 h incubation with neurotransmitters elicited an increase in NBT reduction in BV2 microglia, in line with the optimised protocol. The data (Fig. 3.1D) show that 4 h incubation of BV2 microglia with glutamate (1 µM), GABA (100 µM) or BzATP (250 µM), induced a significant increase in NBT reduction (*$p<0.05$). However, at 16 h incubation with glutamate, superoxide production was also significantly elevated (***$p<0.001$). To maintain consistency with the assay however, all readings were taken at the 4 h time point (Fig,3.1D).

It was next important to determine whether glutamate, GABA or BzATP induced superoxide production (shown by NBT reduction) in BV2 microglia was a consequence of NADPH oxidase activity. BV2 microglia were treated with each neurotransmitter in the presence of the specific NADPH oxidase inhibitor apocynin (10 µM), and superoxide production was assessed by NBT reduction (Fig. 3.1E). Treatment of BV2 microglia with the neurotransmitters glutamate, GABA or BzATP in the presence of apocynin significantly decreased NBT reduction and therefore superoxide production. This suggests that glutamate, GABA and BzATP induce superoxide production through activation of the NADPH oxidase.

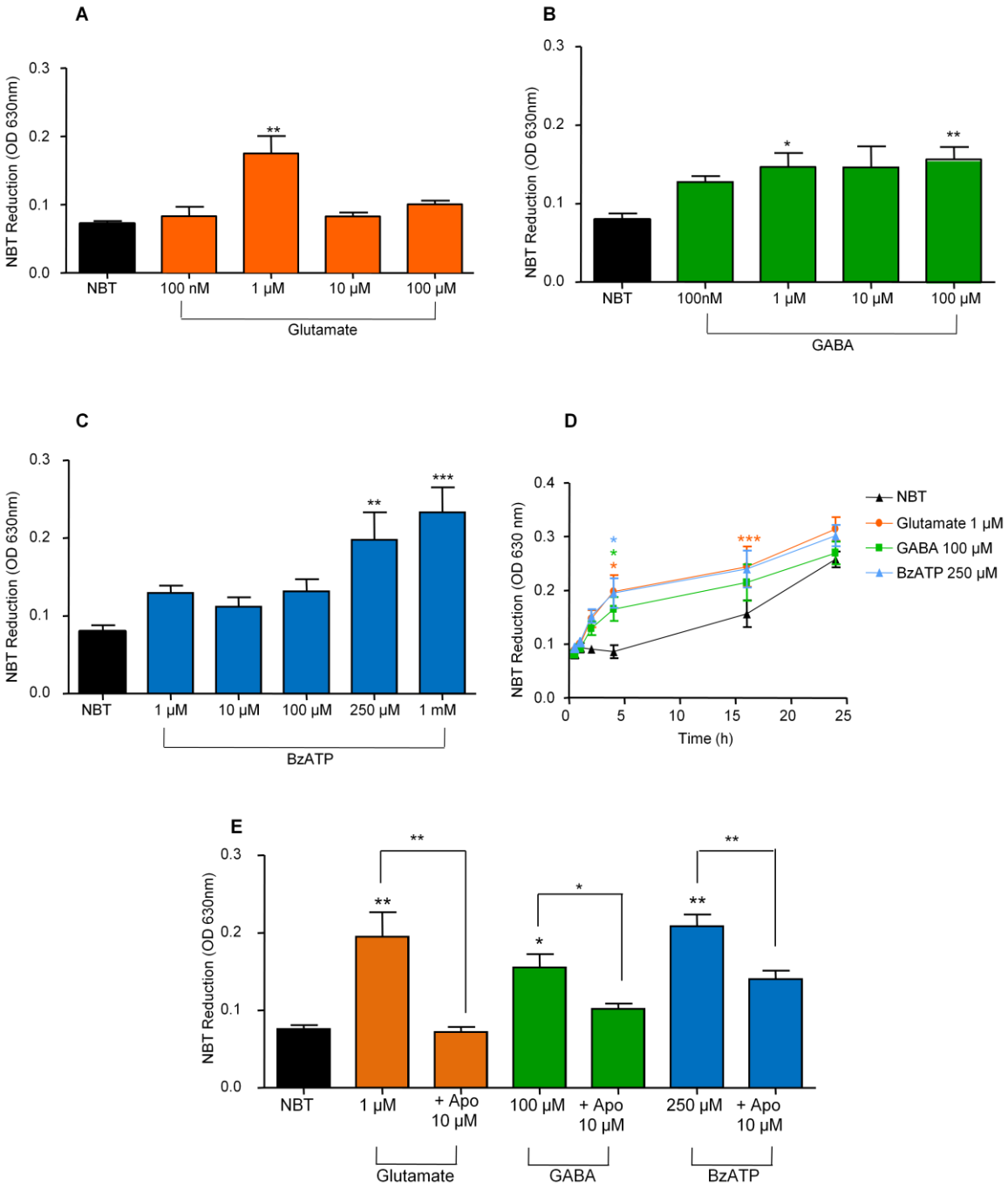

Figure 3.1 NBT analysis showing induction of superoxide production by neurotransmitters. *BV2 cells were treated with the neurotransmitters glutamate 100 nM, 1µM, 10 µM or 100 µM* ***(A)****, GABA 100 nM, 1µM, 10 µM or 100 µM* ***(B)****, or BzATP 1µM, 10 µM, 100 µM, 250 µM or 1 mM* ***(C)****, to determine the optimal concentration of each neurotransmitter required to induce a significant increase in NBT reduction when compared to basal NBT reduction in un-treated BV2 microglia. BV2 microglia were also treated with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence of NBT for 30 min, 1 h , 4 h, 8 h , 16 h and 24 h to validate the use of the 4 h time point in the NBT assay* ***(D).*** *To determine whether NBT reduction following treatment with glutamate, GABA or BzATP was a consequence of the NADPH oxidase, BV2 microglia were co-treated with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence of the NADPH oxidase inhibitor apocynin (10 µM)* ***(E).*** *Data were analysed using one way ANOVA with Tukey post-hoc analysis, and comparisons were made between treatments and basal superoxide production (ie. NBT) and also between treatment with the neurotransmitters and cells treated with the neurotransmitters and apocynin as appropriate. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

It was important to determine whether these findings could be replicated in primary microglia. The NBT assay was not suitable for use with primary microglia, as primary microglia could not to grow in 96 well plates directly on plastic and at the low cell density required. However the fluorescent probe dHEth was used to investigate the superoxide production in primary microglia using fluorescence microscopy. Microglia plated on glass coverslips were treated with glutamate (1 μM), GABA (100 μM) or BzATP (250 μM) either alone or in the presence of apocynin (10 μM), for 24 h (Fig. 3.2A and B) as determined by optimisation of the assay. The data (Fig. 3.2A) shows that treatment with glutamate, GABA or BzATP significantly increased the number of superoxide positive microglia, which could be significantly attenuated by co-treatment with the NADPH oxidase inhibitor apocynin (representative images are shown in Fig. 3.2B), therefore treatment of primary microglia with the neurotransmitters glutamate, GABA or BzATP induces superoxide production in an NADPH oxidase dependent manner.

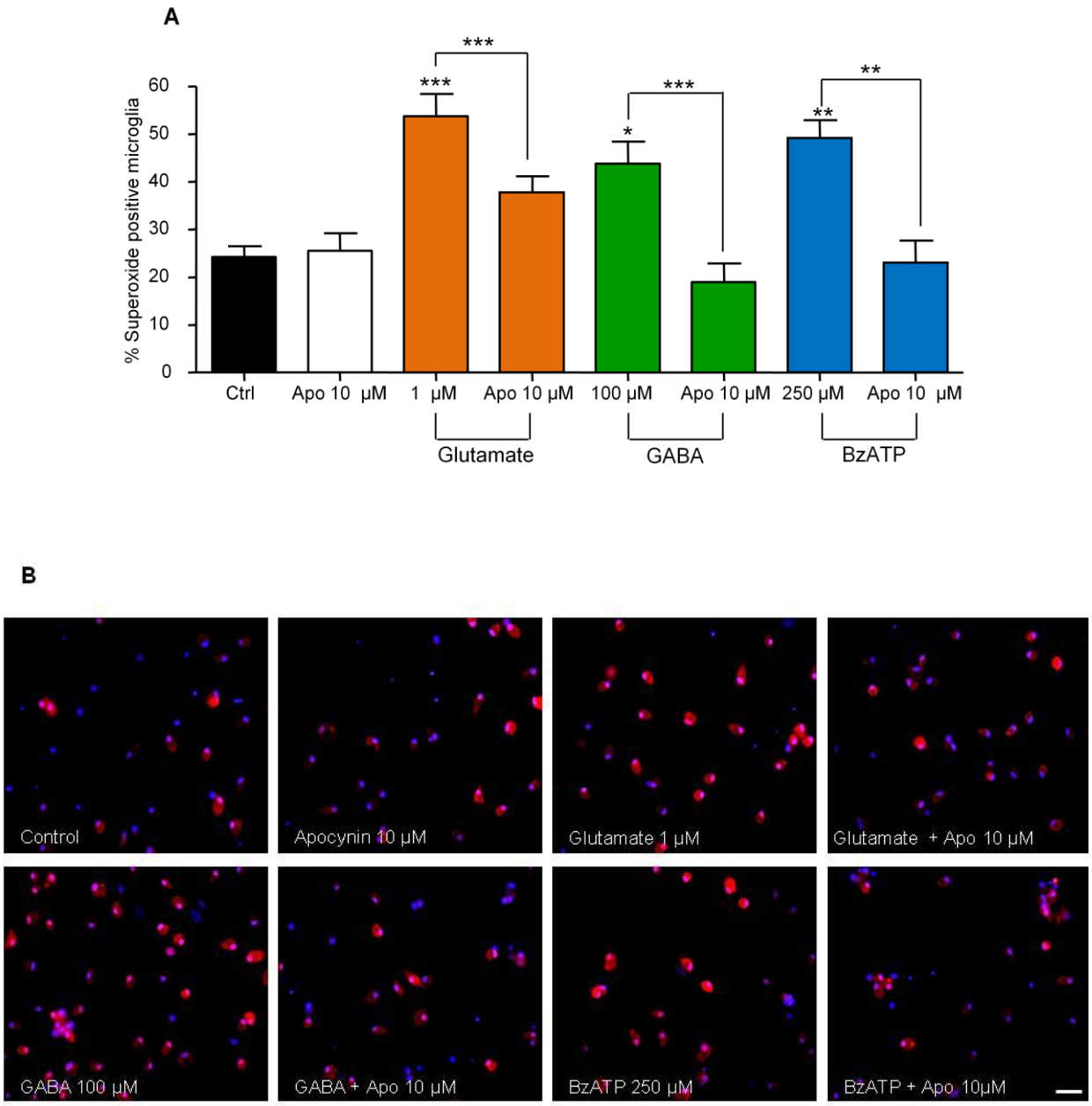

Figure 3.2 Neurotransmitters induce microglial superoxide production – analysis by dHEth fluorescence imaging. *Microglia plated on glass coverslips were treated with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) either alone or in combination with apocynin (10 µM) for 24 h in line with the assay optimisation. Data in* ***(A)*** *shows the percent superoxide positive cells across three experiments, assessed by red fluorescence in the nuclei, and* ***(B)*** *shows representative images. Data were analysed using one way ANOVA with Tukey post-hoc analysis. Each condition was compared with basal superoxide production in control untreated cells, and cells treated with apocynin were compared with their corresponding condition. *p<0.05, **p<0.01, ***p<0.001. Data are n=3.Scale bar 20 µm.*

As the dHEth fluorescence microscopy data are semi-quantitative, flow cytometry and HPLC analysis were used to quantify the imaging data showing that glutamate, GABA and BzATP induced superoxide production in an NADPH oxidase dependent manner. Due to the large numbers of cells required, and the fact that primary microglia can be activated by detachment from culture vessels, which can induce a change in morphology and the production of activation markers such as superoxide; BV2 microglia were used for these analyses. Cells were treated with the neurotransmitters in the presence or absence of apocynin for 24 h in line with the use of the probe in primary microglial cells.

For flow cytometry (Fig. 3.3A, B, C, D), BV2 microglia were treated with glutamate (1 µM), GABA (100 µM), or BzATP (250 µM) in the presence or absence of apocynin (10 µM) for 24 h. Dihydroethidium fluorescence was detected following incubation of cells with the probe for 40 min, and the data were analysed by calculating the mean fluorescence intensity of the population, enabling a quantitative analysis of the total cell population. A significant increase in mean fluorescence intensity was seen upon treatment of BV2 microglia with glutamate (1 µM), GABA (100 µM) or BzATP (250 µM), when compared with untreated controls labelled with dHEth (basal superoxide production), which could be attenuated by co-treatment with apocynin, however, the increase in dHEth fluorescence induced by GABA treatment was not significantly attenuated by apocynin (Fig. 3.3A). GABA may therefore induce the activation of other NADPH oxidase isoforms which may be insensitive to apocynin, or GABA may induce superoxide production through other oxidase systems.

HPLC analysis was also used to quantify superoxide production following treatment with the neurotransmitters glutamate, GABA or BzATP (Fig. 3.3E). Cells were treated for 24 h with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence or absence of apocynin (10 µM), and were incubated with dHEth for 40 min before analysis. The production of 2-OH-E^+, the superoxide specific oxidation product of dHEth,

and the production of the H_2O_2 oxidation product ethidium (Eth), was measured (Fig. 3.3E). Incubation of untreated cells with dHEth showed that basal levels of 2-OH-E^+ and Eth production were low, and treatment of cells with apocynin had little effect on ROS production. Treatment with glutamate (1 µM) or BzATP (250 µM) significantly increased 2-OH-E^+ levels when compared with untreated controls, which could be significantly reduced upon co-treatment with apocynin, suggesting that glutamate and BzATP induce superoxide production in an NADPH oxidase dependent manner. Treatment of BV2 microglia with GABA (100 µM) significantly elevated Eth production when compared with untreated controls, which was also significantly higher than the 2-OH-E^+ produced. Furthermore, Eth production was significantly reduced following co-treatment of cells with apocynin, suggesting that GABA induces the production of ROS rather than superoxide through the NADPH oxidase.

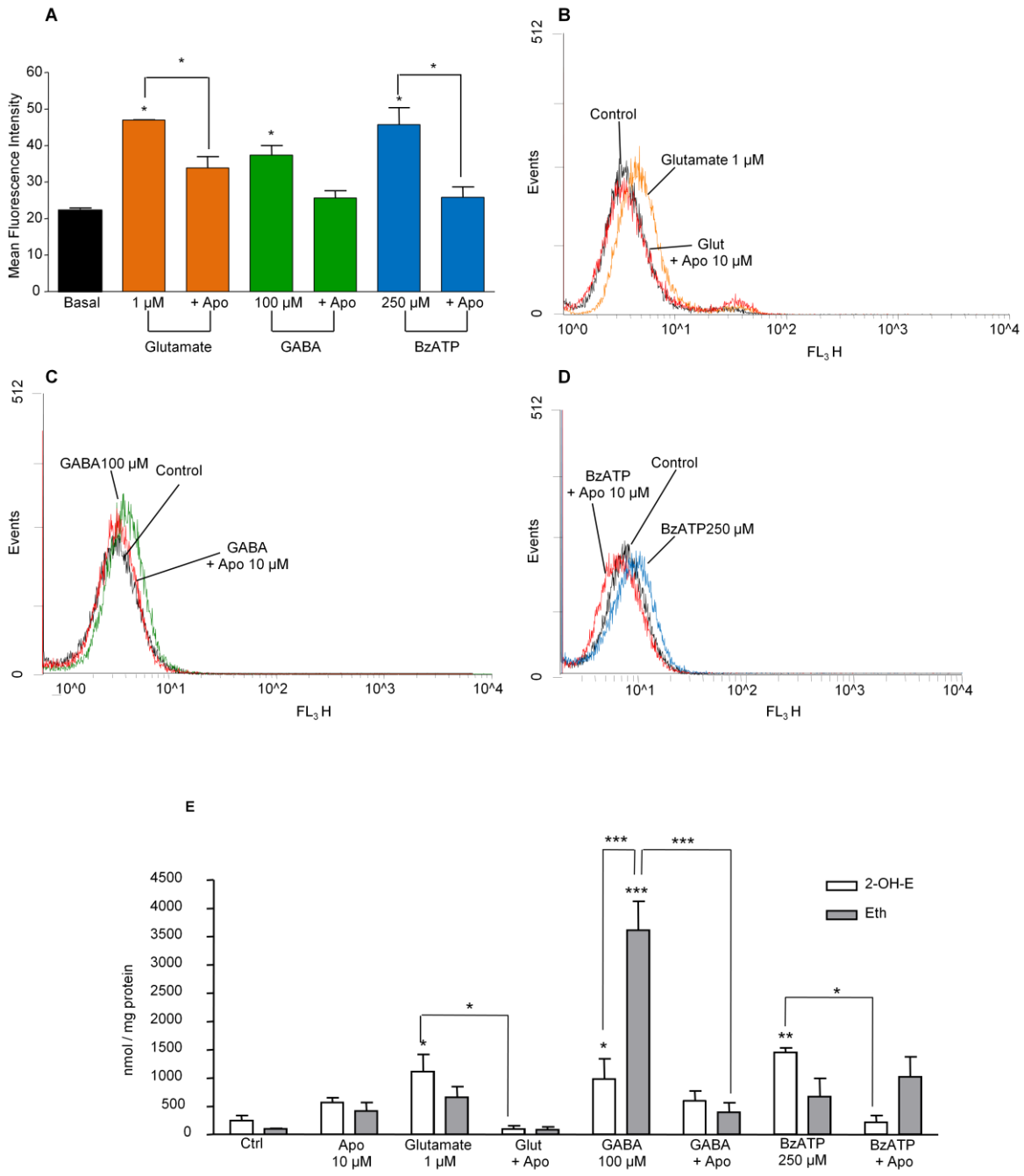

Figure 3.3 Quantification of neurotransmitter induced superoxide production by flow cytometry measuring dHEth fluorescence and analysis of dHEth oxidation by HPLC. *BV2 microglia were treated for 24 h with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence or absence of apocynin (10 µM). Cells were analysed for dHEth fluorescence by flow cytometry, counting 30,000 events. The mean fluorescence intensity for each condition was analysed and is shown in* ***(A).*** *Representative histograms are shown for glutamate* ***(B)****, GABA* ***(C)*** *and BzATP* ***(D)****, and show the fluorescence of treatment with neurotransmitters alone and in combination with apocynin (denoted by the red histogram). Data were analysed by one way ANOVA and Tukey post-hoc analysis. *p<0.05. BV2 microglia were also analysed for superoxide production by HPLC* ***(E).*** *Cells were treated as described in the presence or absence of apocynin for 24 h. The production of the superoxide specific oxidation product 2-OH-E was measured as was the production of the H_2O_2 specific product Eth. Data were analysed by Student's T-Test and also by two way ANOVA for comparisons between 2-OH-E and Eth data. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

These quantitative analyses using the dHEth probe as a sensor for superoxide production show that glutamate (1 µM) and BzATP (250 µM) induce superoxide production in an NADPH oxidase dependent manner. Treatment with GABA (100 µM) induces ROS production rather than superoxide specifically (as shown by HPLC), which was attenuated by co-treatment of cells with apocynin, suggesting that GABA induces NADPH oxidase activation, although may activate other NADPH oxidase isoforms.

Following the findings that microglia produce superoxide after treatment with neurotransmitters in an NADPH oxidase dependent manner, it was important to confirm that superoxide production was a result of NADPH oxidase enzymatic activity. BV2 microglia were used to investigate NADPH oxidase activity, and cells were treated with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence or absence of apocynin (10 µM) for 4 h. The data (Fig. 3.4) show that treatment with glutamate or GABA significantly increased NADPH oxidase activity, which could be reduced to basal levels by co-treatment of cells with apocynin. However, BzATP did not significantly increase NADPH oxidase activity, although co-treatment with apocynin attenuated enzymatic activity, thereby suggesting that BzATP may mediate the activation of the NADPH oxidase and other oxidase systems.

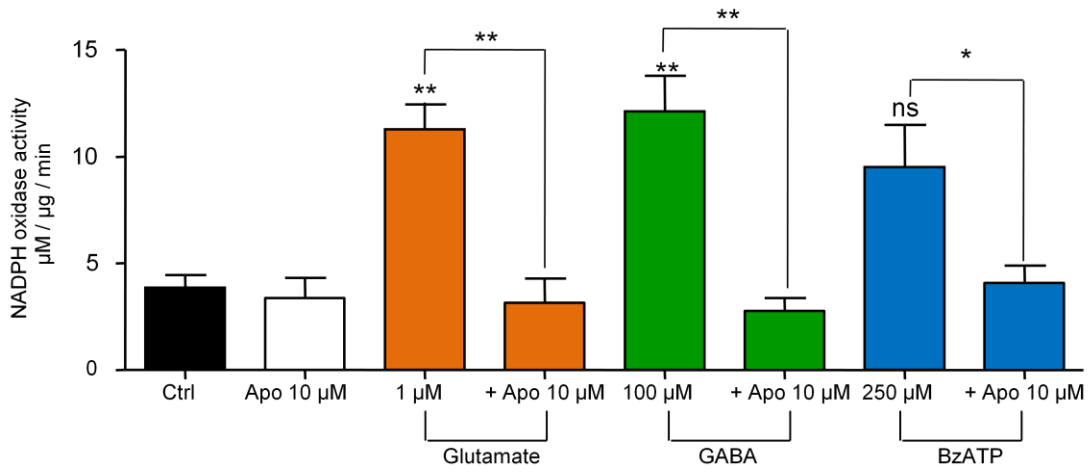

Figure 3.4 NADPH oxidase activity following treatment with neurotransmitters. *BV2 microglia were treated with glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence or absence of apocynin (10 µM) for 4 h. The reduction of NADPH to NADP was followed over 10 min at 340 nm, and the reduction of NADPH was normalized to protein content. Data were analysed by one way ANOVA and Tukey post-hoc analysis was performed, in which all conditions were compared with contro untreated cells, and the treatments plus apocynin were compared with their respective treatment groups.*p<0.05, **p<0.01. Data are n=3.*

Together these data show that superoxide production through NADPH oxidase activity can be induced by the neurotransmitters glutamate, GABA and BzATP in microglia. Following these findings, it was important to determine which neurotransmitter receptors glutamate, GABA or ATP were acting through to induce NADPH oxidase activation, to investigate the mechanisms involved in superoxide production, and to learn more about the signalling pathways leading to activation of the NADPH oxidase following exposure of microglia to neurotransmitters.

3.2.2 Neurotransmitter receptor agonists and antagonists induce superoxide production in microglia as a consequence of NADPH oxidase activation

As glutamate, GABA and BzATP induced superoxide production through NADPH oxidase activation, superoxide production through modulation of mGluRs, iGluRs, GABA or purinergic receptors were investigated. In each case, the NBT assay was used to test whether a wide variety of receptor agonists or antagonists could modulate superoxide production, before the more sensitive assays (dHEth imaging, HPLC and flow cytometry) were used to investigate positive results in more detail.

3.2.2.1 Modulation of mGluRs induces superoxide production in microglia

Group I mGluR agonists reduce NADPH oxidase expression in microglia *in vivo* after spinal cord damage (Byrnes et al. 2009), which is protective through the inhibition of ROS production and microglial reactivity (Loane et al. 2009). Here, group I, II and III mGluRs were modulated to investigate the production of NADPH oxidase dependent superoxide in microglia. Initially, an NBT assay was performed to investigate whether modulation of the group I, II or III mGluRs could induce NBT reduction suggesting superoxide production (Fig. 3.5). BV2 microglia were treated with the group I agonists DHPG (100 µM) (Palmer et al. 1997), an activator of mGluR1 and mGluR5, or CDPPB (500 nM) (Kinney et al. 2005) a selective activator of mGluR5; and the antagonists AIDA (250 µM) (Melendez et al. 2005) an

mGluR1a antagonist, or MTEP (100 nM) (Cosford et al. 2003), a selective mGluR5 antagonist (Fig. 3.5A). Treatment with CDPPB (500 nM) or MTEP (100 nM) significantly increased NBT reduction (Fig. 3.5A). NBT reduction was also measured following treatment of BV2 microglia with the group II agonists DCG-IV (500 nM) (Cartmell et al. 1998) or NAAG (50 µM) (Wroblewska et al. 1997) a highly selective mGluR3 agonist (Neale et al. 2000); and the antagonist APICA (250 µM) (Zhou et al. 2006) (Fig. 3.5B). Treatment with the selective mGluR3 agonist NAAG significantly increased NBT reduction in BV2 microglia (Fig. 3.5B). The NBT assay was also performed following treatment of BV2 microglia with the group III agonist L-AP4 (100 µM) (Zhou et al. 2006) and antagonist MAP4 (500 µM) (Faden et al. 1997) (Fig. 3.5C), with a significant increase in NBT reduction observed following treatment with L-AP4 (Fig. 3.5C).

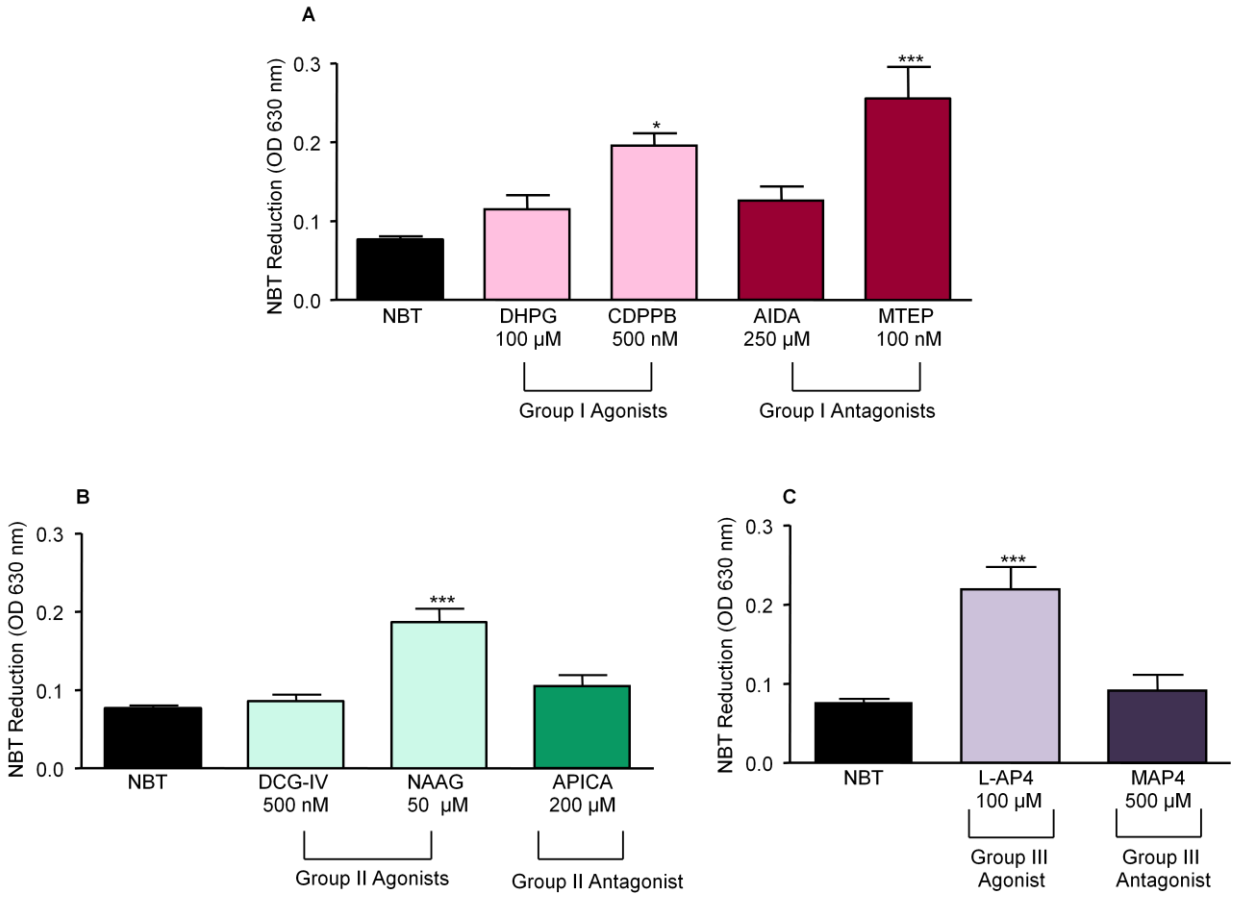

Figure 3.5 NBT assay to investigate superoxide production following treatment of BV2 microglia with (A) group I, (B) group II and (C) group III mGluR agonists and antagonists. *BV2 microglia were treated with the group I mGluR agonists DHPG (100 µM) or CDPPB (500 nM), or antagonists AIDA (250 µM) or MTEP (100 nM)* ***(A)****; the group II agonists DCG-IV (500 nM) or NAAG (50 µM) or antagonist APICA (200 µM)* ***(B)****; or the group III agonist L-AP4 (100 µM) or the antagonist MAP4 (500 µ)* ***(C).*** *BV2 microglia were analysed for NBT reduction, which was measured after 4 h treatment. Data were analysed using one way ANOVA, in which all conditions were compared with the NBT only control (basal NBT reduction). *p<0.05, ***p<0.001. All data are n=3.*

These data show that inhibition of the group I mGluR with MTEP, activation of the group II mGluR3 with NAAG and activation of the group III mGluR with L-AP4 induces superoxide production in BV2 microglia (Fig. 3.5). To investigate this further, a dose dependency analysis of MTEP, NAAG and L-AP4 was performed (Fig. 3.6) to determine the optimal concentration of each mGluR modulator required to significantly increase NBT reduction, and therefore induce superoxide production in BV2 microglia. Treatment of BV2 microglia with MTEP at a concentration of 100 nM significantly increased NBT reduction (Fig. 3.6A), whilst NAAG used at 5 µM or 50 µM increased NBT reduction (Fig. 3.6B), and treatment with L-AP4 at 10 µM or 100 µM also significantly increased NBT reduction (Fig. 3.6C). Following these dose-dependency NBT analyses, the group I antagonist MTEP was used in all later experiments at 100 nM, whilst NAAG was used at 50 µM, as this higher concentration acts specifically at the mGluR3 receptor (Wroblewska et al. 1997); and L-AP4 was used at 100 µM, which has been shown in the literature to induce receptor activation (Zhou et al. 2006). Furthermore, both NAAG at 50 µM (***$p<0.001$) and L-AP4 at 100 µM (***$p<0.001$) induced a more significant increase in NBT reduction when compared with their use at a lower concentration, suggesting that these are the optimal concentrations required for superoxide production.

Following the findings that MTEP at 100 nM, NAAG at 50 µM and L-AP4 at 100 µM induced a significant increase in NBT reduction, it was important to determine whether this was a consequence of NADPH oxidase activation. BV2 microglia were treated with MTEP (100 nM), NAAG (50 µM) or L-AP4 (100 µM) in the presence or absence of apocynin (10 µM), and superoxide production was assessed by NBT reduction (Fig. 3.6D). NBT reduction following modulation of the mGluRs was attenuated by co-treatment with apocynin, showing that inhibition of the group I mGluR, and activation of mGluR3 and the group III mGluRs induced NADPH oxidase activation.

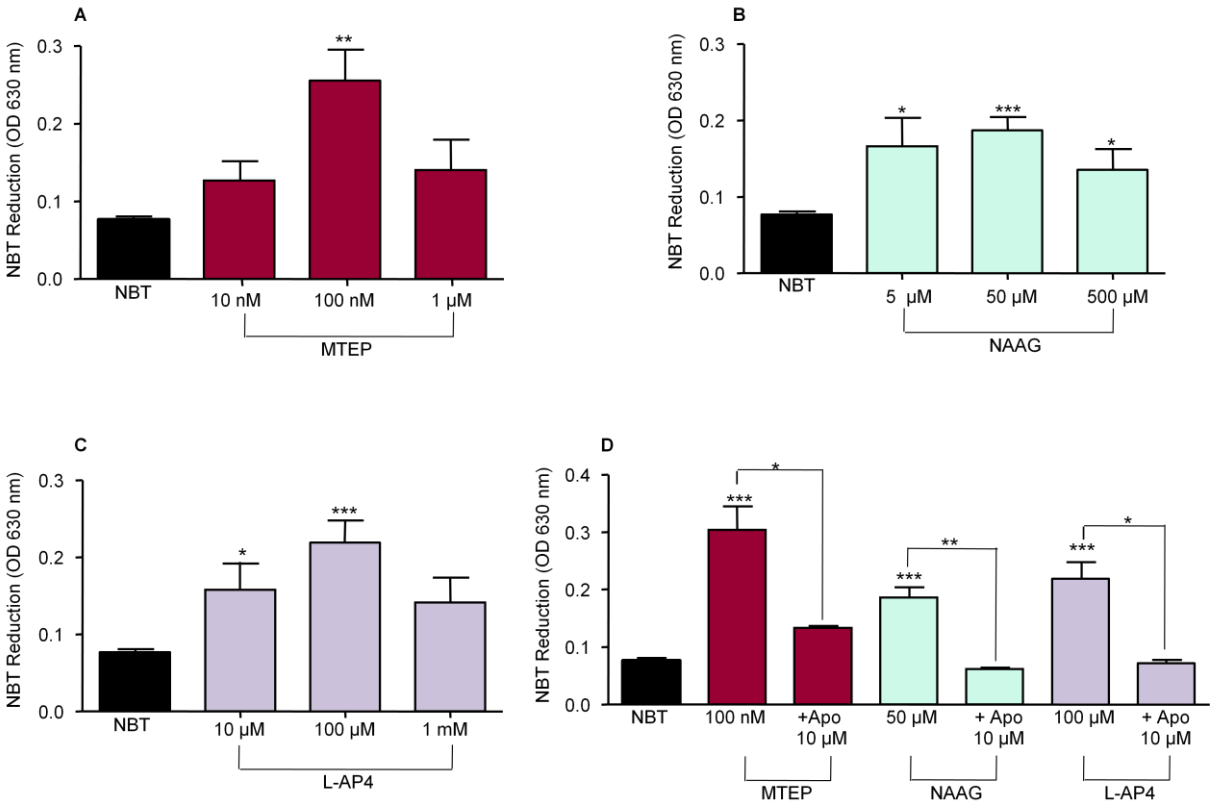

Figure 3.6 Dose-dependency NBT analysis of MTEP (A), NAAG (B) and L-AP4 (C) to determine the optimal concentration required to induce superoxide production in an NADPH oxidase dependent manner. *BV2 microglia were treated with MTEP 10 nM, 100 nM or 1 µM* ***(A)****; NAAG 5 µM. 50 µM or 500 µM* ***(B)****; L-AP4 10 µM, 100 µM or 1 mM* ***(C)*** *for 4 h before measurement of NBT reduction. Statistical analysis was performed using a one way ANOVA with Tukey post hoc analysis, in which each condition was compared with the NBT only control (basal superoxide production). The involvement of the NADPH oxidase was then confirmed with the use of apocynin* ***(D)****. BV2 microglia were treated with MTEP (100nM), NAAG (50 µM) or L-AP4 (100 µM) in the presence or absence of the NADPH oxidase inhibitor apocynin (10 µM) for 4 h before NBT reduction was analysed at 630 nm. Statistical analysis was performed as described, with comparisons being made between all conditions and the NBT only control, and also between each treatment in the presence or absence of apocynin. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

The findings that treatment of BV2 microglia with MTEP, L-AP4 or NAAG induced superoxide production in a NADPH oxidase dependent manner was replicated in primary microglia using fluorescence imaging of dHEth (Fig. 3.7). Primary microglia were treated with MTEP (100 nM), NAAG (50 μM) or L-AP4 (100 μM) in the presence or absence of apocynin for 24 h before cells were incubated with dHEth and imaged for red fluorescence in the nuclei (Fig. 3.7B). Inhibition of the group I mGluR, and activation of mGluR3 and the group III mGluRs induced NADPH oxidase dependent superoxide production, as dHEth fluorescence could be attenuated by co-treatment with the NADPH oxidase inhibitor apocynin (Fig. 3.7). This imaging data is therefore in agreement with the NBT analysis.

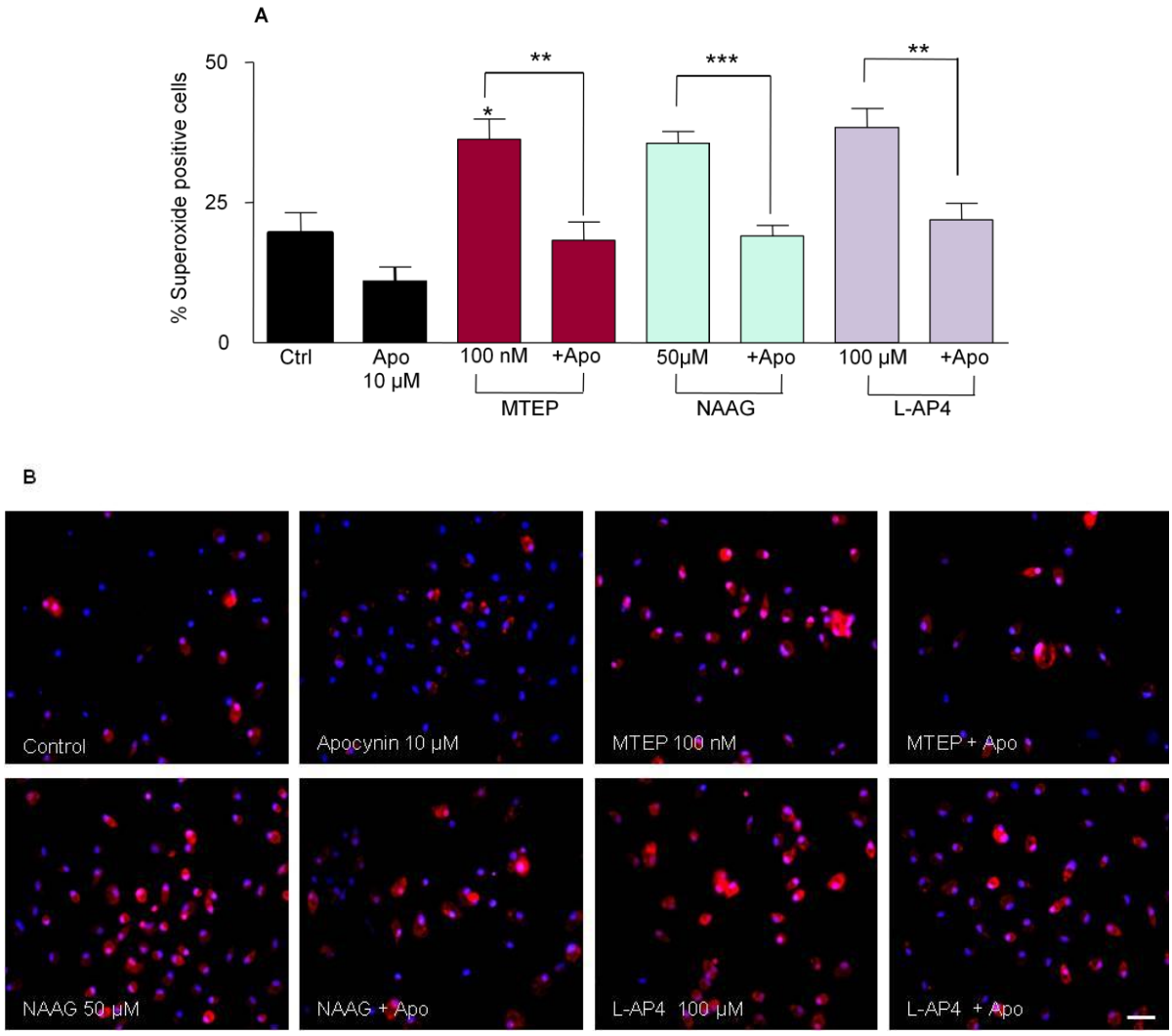

Figure 3.7 dHEth fluorescence showing NADPH oxidase dependent superoxide production in primary microglia following treatment with MTEP, NAAG or L-AP4. *Primary microglia were plated on glass coverslips at 50,000 cells per well and were used within 24 h of isolation. Microglia were treated with the mGluR modulators MTEP (100 nM), NAAG (50 µM) or L-AP4 (100 µM) in the presence or absence of the NADPH oxidase inhibitor apocynin (10 µM). Dihydroethidium fluorescence was then assessed by counting the number of cells exhibiting red fluorescence in the nuclei* ***(A).*** *Representative images are shown in* ***(B).*** *Statistical analysis was performed using a one way ANOVA with Tukey post-hoc analysis. All conditions were compared with untreated controls (basal superoxide production), and each treatment was compared to the respective treatment plus apocynin. *p<0.05, **p<0.01, ***p<0.001. All data are n=3. Scale bar 20 µm.*

Quantitative analysis of mGluR induced dHEth fluorescence was performed using flow cytometry and HPLC (Fig, 3.8). BV2 microglia were treated with the mGluR modulators MTEP (100 nM), NAAG (50 µM) or L-AP4 (100 µM) in the presence or absence of apocynin (10 µM) for 24 h, and cells were incubated with dHEth for 40 min before flow cytometry (Fig. 3.8A, B, C, D) or HPLC (Fig. 3.8E) were performed. The flow cytometry data was in agreement with both the fluorescence imaging and NBT analysis, revealing that L-AP4 (Fig. 3.8A, B), NAAG (Fig. 3.8A, C) and MTEP (Fig. 3.8A, D) significantly increased mean dHEth fluorescence intensity which was attenuated by co-treatment with apocynin (Fig. 3.8A). This therefore provides further support to the previous findings that modulation of the microglial mGluRs induces superoxide production in an NADPH oxidase dependent manner.

Quantitative analysis of dHEth oxidation products by HPLC (Fig. 3.8E) also correlated with the flow cytometry and imaging data. Treatment of BV2 microglia with MTEP (100 nM) or L-AP4 (100 µM) significantly elevated the production of the superoxide specific product 2-OH-E^+ when compared with control levels, which could be significantly reduced by co-treatment with apocynin, suggesting that MTEP and L-AP4 induced superoxide production was through activation of the NADPH oxidase. MTEP or L-AP4 did not significantly modulate Eth production, suggesting that inhibition of the group I mGluRs and activation of the group III mGluRs induce superoxide production specifically in an NADPH oxidase dependent manner. Treatment of BV2 microglia with NAAG (50 µM) did not significantly elevate 2-OH-E^+ or Eth production. As neither 2-OH-E^+ nor Eth production was significantly increased when compared with controls, it could be suggested that NAAG induces the production of low levels of ROS possibly through different Nox isoforms.

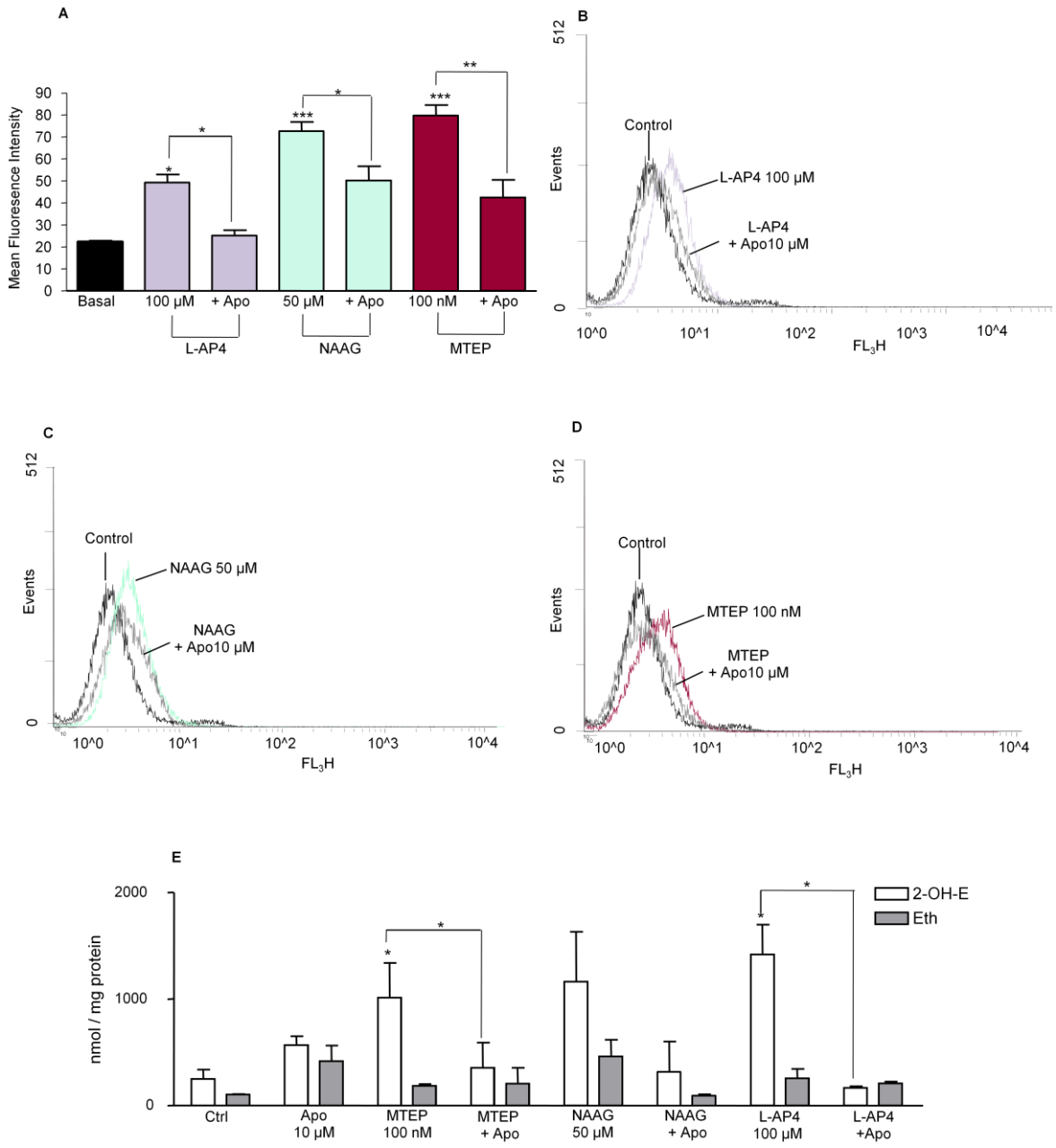

Figure 3.8 Quantitative analysis of superoxide production following modulation of mGluR's by flow cytometry analysis of dHEth fluorescence and HPLC analysis of dHEth oxidation products. *For flow cytometry analysis of dHEth fluorescence, BV2 microglia were treated with the group III activator L-AP4 (100 µM)* ***(B)****, the mGluR3 agonist NAAG (50 µM)* ***(C)*** *or the group I antagonist MTEP (100 nM)* ***(D)*** *in the presence or absence of the NADPH oxidase inhibitor apocynin (10 µM) (denoted with a gray line on the representative histograms). The histograms show that each treatment induces a shift in the dHEth fluorescence which can be attenuated by co-treatment with apocynin. The mean fluorescence intensity* ***(A)*** *was calculated and analysed using a one way ANOVA with Tukey post-hoc analysis, in which all treatments were compared with basal dHEth fluorescence and comparisons were also made between treatments with and without apocynin. *p<0.05, **p<0.01, ***p<0.001. HPLC analysis* ***(E)*** *was performed on cells treated with the receptor agonists or antagonists in the presence or absence of apocynin for 24 h. Cells were then analysed for 2-OH-E and Eth production to detect superoxide and H_2O_2 production respectively. Data were analysed by Student's T-Test and two way ANOVA to compare 2-OH-E and Eth production. *p<0.05. All data are n=3.*

Investigation into the enzymatic activity of the NADPH oxidase following treatment with the mGluR modulators also agreed with the analysis of superoxide production (Fig. 3.9). The NADPH oxidase activity assay revealed that treatment with MTEP (100 nM), NAAG (50 µM) or L-AP4 (100 µM) significantly increased NADPH oxidase activity when compared with control untreated cells (basal NADPH oxidase activity). Furthermore, co-treatment of cells with each mGluR modulator and the NADPH oxidase inhibitor apocynin attenuated NADPH oxidase activity, lending support to the previous findings that superoxide production following inhibition of the group I mGluR with MTEP, and activation of mGluR3 or the group III mGluRs is through activation of the NADPH oxidase.

Together these data show that inhibition of the group I mGluRs and activation of mGluR3 and the group III mGluRs induce superoxide production in an NADPH oxidase dependent manner.

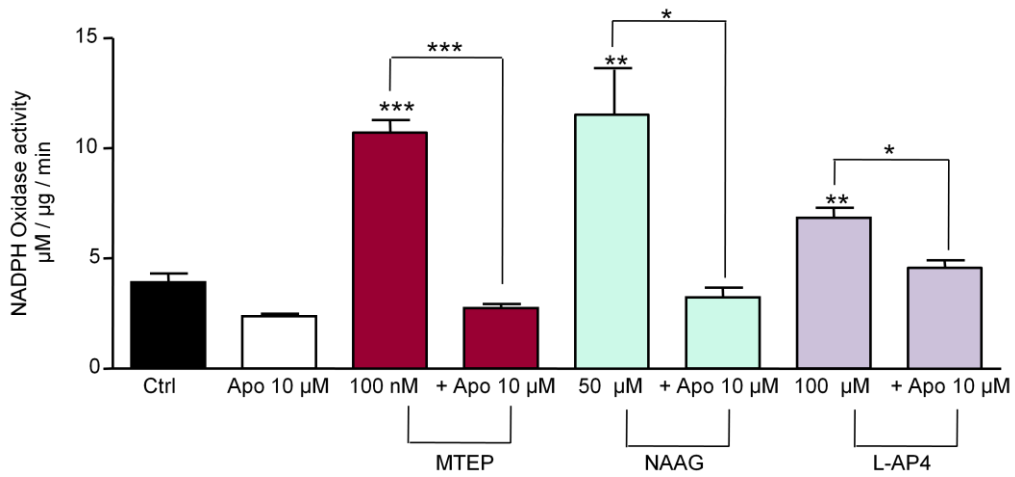

Figure 3.9 Modulation of mGluR's induces NADPH oxidase activity. *BV2 microglia were treated with the mGluR modulators MTEP (100 nM), NAAG (50 µM) or L-AP4 100 µM) in the presence or absence of apocynin (10 µM) for 4 h before analysis of NADPH oxidase activity. Enzymatic activity was normalized to protein content. Statistical analysis was performed on normalized data using a one way ANOVA, and comparisons were made between treated cells and control untreated cells (basal NADPH oxidase activity), and also between cells treated with modulators alone and those co-treated with apocynin. *p<0.05, **p<0.01, ***p<0.001. Data are n=3.*

3.2.2.2 Modulation iGluRs induces superoxide production in microglia

It was next important to determine whether iGluR modulation could mediate microglial superoxide production. Microglia express subunits of the AMPA and Kainate receptors, with activation of each receptor subtype inducing an inward current seen by electrophysiology, (Noda et al. 2000), which could be attenuated by co-treatment with receptor antagonists, suggesting the presence of functional receptors on microglia (Hagino et al. 2004). Furthermore, microglia express all AMPA receptor subunits (GluR1-4), lending further support to the presence of functional receptors on microglia (Hagino et al. 2004). Recent *in vivo* studies have shown that microglia express functional NMDA receptors, demonstrated by the presence of an enhanced calcium influx following administration of NMDA which could be inhibited by co-treatment of microglia with the antagonist MK-801 (Murugan et al. 2011). Furthermore, microglia express the NMDA receptor subunits NR1, NR2A-D and NR3A, which are up-regulated after ischaemia (Murugan et al. 2011). In addition, modulation of the microglial NMDA receptor induces NO production, suggesting that modulation of the NMDA receptor induces microglial activation (Murugan et al. 2011).

The involvement of AMPA and NMDA receptor modulation in microglial superoxide production and NADPH oxidase activation was investigated here. BV2 microglia were assessed for superoxide production using the NBT assay. Cells were treated with NMDA (100 µM) (Liang et al. 2010) or MK-801 (10 µM) (Murugan et al. 2011) either alone or in combination with apocynin (10µM) (Fig. 3.10Ai); or were treated with the AMPA receptor agonist Quisqualic Acid (QA) (10 µM) (Noda et al. 2000) or the AMPA receptor antagonist CNQX (10 µM) (Noda et al. 2000) either alone or in combination with apocynin (10 µM) (Fig. 3.10Aii). Treatment with NMDA significantly increased NBT reduction when compared with basal NBT reduction, which could be attenuated by co-treatment with the NADPH oxidase inhibitor apocynin, suggesting that activation of the microglial NMDA

receptor promotes NADPH oxidase activity (Fig. 3.10Ai). A significant increase in NBT reduction compared with basal levels was also seen following treatment of BV2 microglia with the NMDA receptor antagonist MK-801, however this could not be attenuated by co-treatment of cells with apocynin (Fig. 3.10.Ai), suggesting that inhibition of NMDA receptors on microglia may induce the production of ROS through other oxidase systems. Treatment of BV2 microglia with the AMPA receptor agonist QA did not significantly increase NBT reduction when compared with basal levels (Fig. 3.10Aii); however treatment with the AMPA receptor antagonist CNQX significantly elevated NBT reduction when compared with basal levels (Fig. 3.10Aii). This increase could not be reduced by co-treatment with apocynin, suggesting that CNQX could not activate the NADPH oxidase (Fig. 3.10Aii).

Further investigations were performed using primary microglial cells and dHEth fluorescence (Fig. 3.10Bi,ii and Fig. 3.10Ci, ii) to support the NBT analysis. The imaging showed that treatment of microglia with NMDA (100 µM) significantly increased the percent of dHEth positive microglia (Fig. 3.10Bi and Ci), which was attenuated by co-treatment with apocynin, and also by co-treatment with NMDA and the NMDA receptor antagonist MK-801 (Fig. 3.10Bi and Ci). This therefore suggests that it is activation of the NMDA receptor specifically that induces NADPH oxidase activation, and lends some support to the suggestion that microglia express functional NMDA receptors. Treatment of microglia with the AMPA receptor agonist QA (10 µM) significantly increased the percentage of dHEth positive microglia, however this was not dependent on NADPH oxidase activation (Fig. 3.10Bii and Cii). Furthermore, treatment with the AMPA receptor antagonist CNQX did not induce a significant increase in the number of dHEth positive cells, suggesting that modulation of the AMPA receptor does not affect superoxide production in primary microglia.

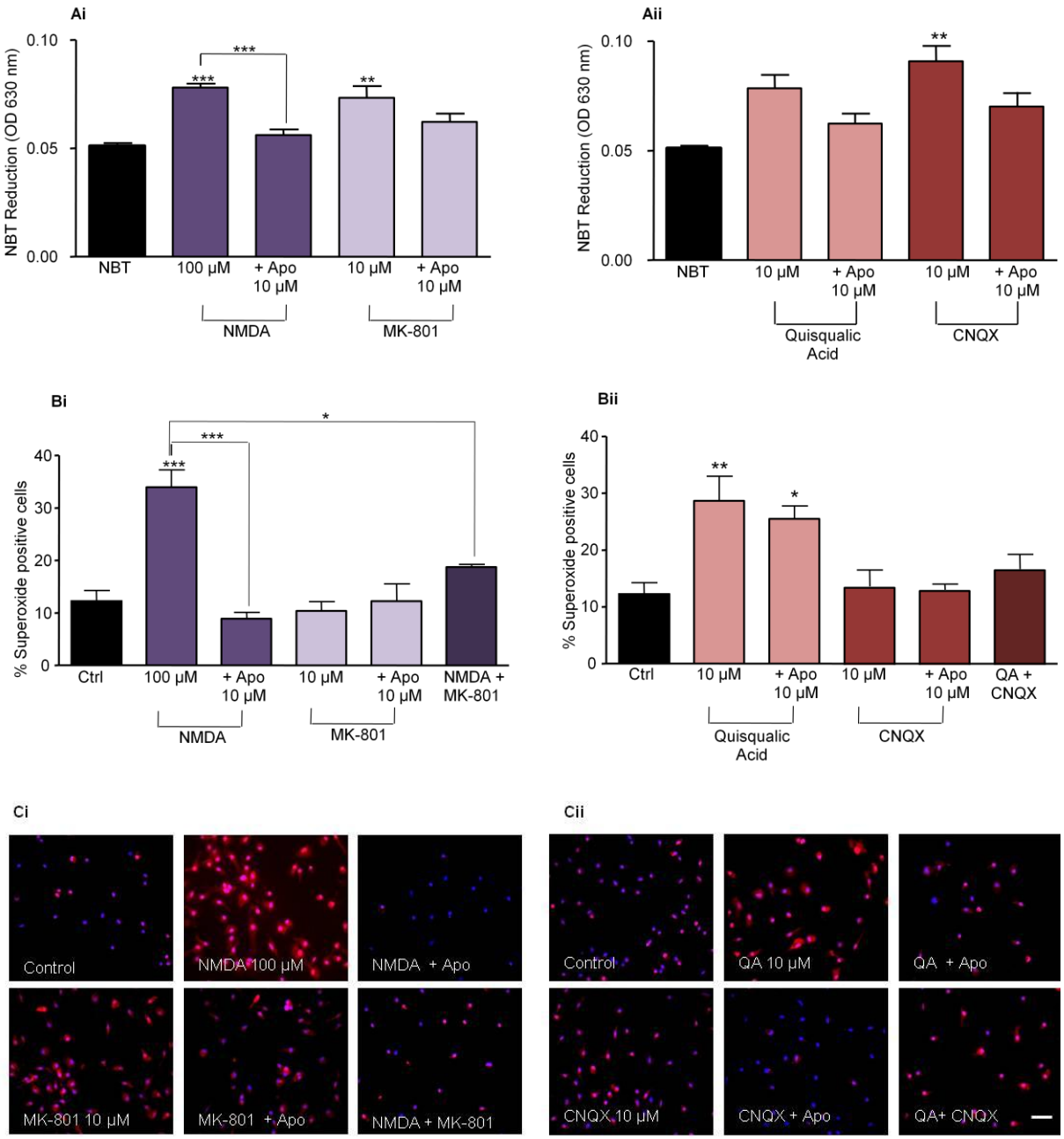

Figure 3.10 Analysis of superoxide production in BV2 cells (Ai, ii) and primary microglia (Bi, ii and Ci, ii) following modulation of the NMDA and AMPA receptors. *BV2 microglia were analysed for superoxide production using the NBT assay* ***(Ai, ii)****. Cells were treated with NMDA (100 µM), MK-801 (10 µM) in the presence or absence of apocynin (10 µM)* ***(Ai)****, or QA (10 µM), CNQX (10 µM) in the presence or absence of apocynin (10 µM). Cells were analysed for NBT reduction after 4 h incubation. Primary microglia were analysed for superoxide production by dHEth fluorescence* ***(Bi, ii and Ci, ii).*** *Primary microglia were treated with NMDA (100 µM) or MK-801 (10 µM) either alone or in combination, or in combination with apocynin for 24 h* ***(Bi, Ci).*** *Primary microglia were also treated with the AMPA agonist QA (10 µM) or the antagonist CNQX (10 µM) either alone or in combination, or in combination with apocynin (10 µM) for 24 h* ***(Bii, Cii).*** *Microglia were considered superoxide positive when exhibiting red fluorescence in the nuclei. All data were analysed using one way ANOVA and Tukey post hoc analysis, making comparisons between basal superoxide production and treatments and also between treatments with and without apocynin. *p<0.05, *p<0.01, ***p<0.001. All data are n=3. Scale bar 20 µm.*

These data (Fig. 3.10) demonstrate that activation of the NMDA receptor promotes microglial superoxide production; whilst modulation of the AMPA receptor has no effect on NADPH oxidase induced superoxide production. This was confirmed using flow cytometry and HPLC analysis (Fig. 3.11). BV2 microglia were treated with NMDA (100 μM) or QA (10 μM) in the presence or absence of apocynin (10 μM) for 24 h. Cells were incubated with dHEth before analysis by flow cytometry or HPLC. The flow cytometry analysis (Fig. 3.11A) showed that treatment with NMDA significantly increased dHEth mean fluorescence intensity, which was attenuated by co-treatment with apocynin (Fig. 3.11A,B). Treatment with QA did not significantly increase mean dHEth fluorescence intensity (Fig, 3.13A, C), in line with the NBT assay and the dHEth fluorescence. HPLC analysis (Fig, 3.11E) showed that treatment with NMDA (100 μM) significantly increased 2-OH-E^+ production when compared with control untreated cells, which was attenuated by co-treatment of cells with the NADPH oxidase inhibitor apocynin. Eth was also increased following treatment with NMDA, and could be decreased upon apocynin co-treatment, however this was not significant, but could point to the involvement of other NADPH oxidase isoforms. Treatment of BV2 microglia with QA had no effect on 2-OH-E^+ or Eth levels, suggesting that modulation of the AMPA receptor does not modulate superoxide production.

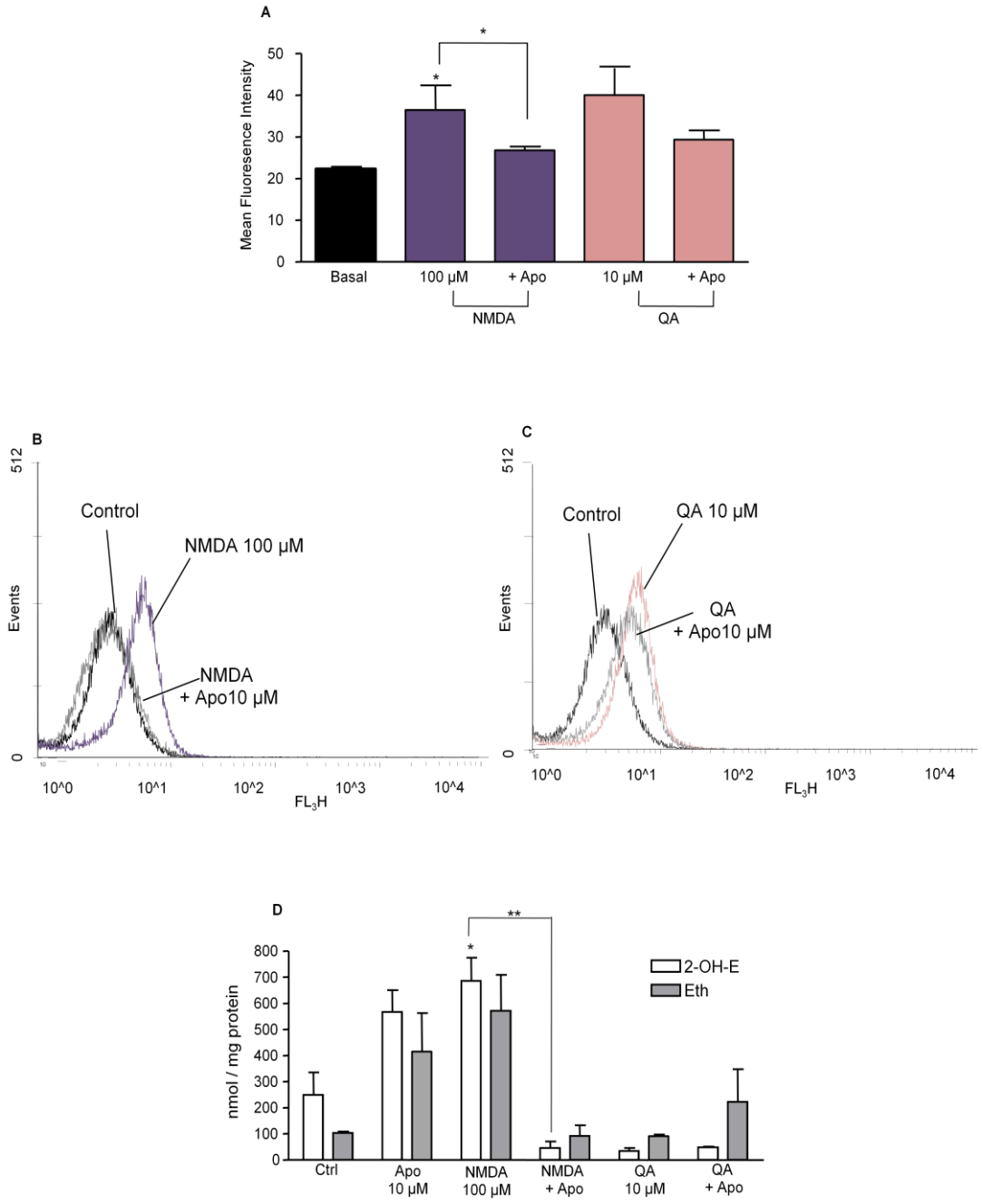

Figure 3.11 Quantative analysis of iGluR induced superoxide production by flow cytometry analysis of dHEth fluorescence and HPLC analysis of dHEth oxidation products. *BV2 microglia were treated with NMDA (100 µM) or QA (10 µM) in the presence or absence of apocynin (10 µM) for 24 h. Cells were then incubated with dHEth before analysis by flow cytometry* ***(A,B,C)*** *or HPLC* ***(D)****. For flow cytometry, cells were analysed for mean fluorescence intensity of dHEth fluorescence* ***(A)*** *Representative histograms are shown in* ***(B)*** *and* ***(C)*** *for NMDA or QA treatment respectively. Data was analysed by one way ANOVA and Tukey post-hoc analysis, making comparisons between basal superoxide production and treatments, and treatments with and without apocynin. *p<0.05. HPLC analysis* ***(D)*** *was performed to analyse 2-OH-E and Eth production following oxidation of dHEth by NMDA or QA. Data were analysed by Student's T-Test and by two way ANOVA for comparisons between 2-OH-E and Eth.*p<0.05, **p<0.01. All data are n=3.*

The data (Fig. 3.11) confirms that NMDA receptor activation induces superoxide production in an NADPH oxidase dependent manner; whilst modulation of the AMPA receptor does not induce NADPH oxidase mediated superoxide production. To validate these findings the NADPH oxidase activity assay was used. BV2 microglia were treated with NMDA (100 µM) or MK-801 (10 µM) either alone, in combination or in the presence of apocynin (Fig. 3.12A); or with QA (10 µM) or CNQX (10 µM) either alone, in combination, or in the presence of apocynin (Fig. 3.12B). Treatment with NMDA alone induced a significant increase in NADPH oxidase activity when compared with basal activity, in line with the superoxide production analysis, which could be decreased upon co-treatment with apocynin, or upon co-treatment with NMDA and MK-801 (Fig. 3.12A), suggesting that induction of superoxide production and NADPH oxidase activity is a consequence NMDA receptor activation. Treatment with QA or CNQX did not significantly increase NADPH oxidase activity when compared with control untreated cells (Fig. 3.12B), suggesting that modulation of the AMPA receptor cannot induce superoxide production through activation of the NADPH oxidase, in line with the analysis of superoxide production.

Modulation of the microglial NMDA receptor therefore induces superoxide production through activation of the NADPH oxidase, whereas modulation of the AMPA receptor has no effect on NADPH oxidase induced superoxide production or activity.

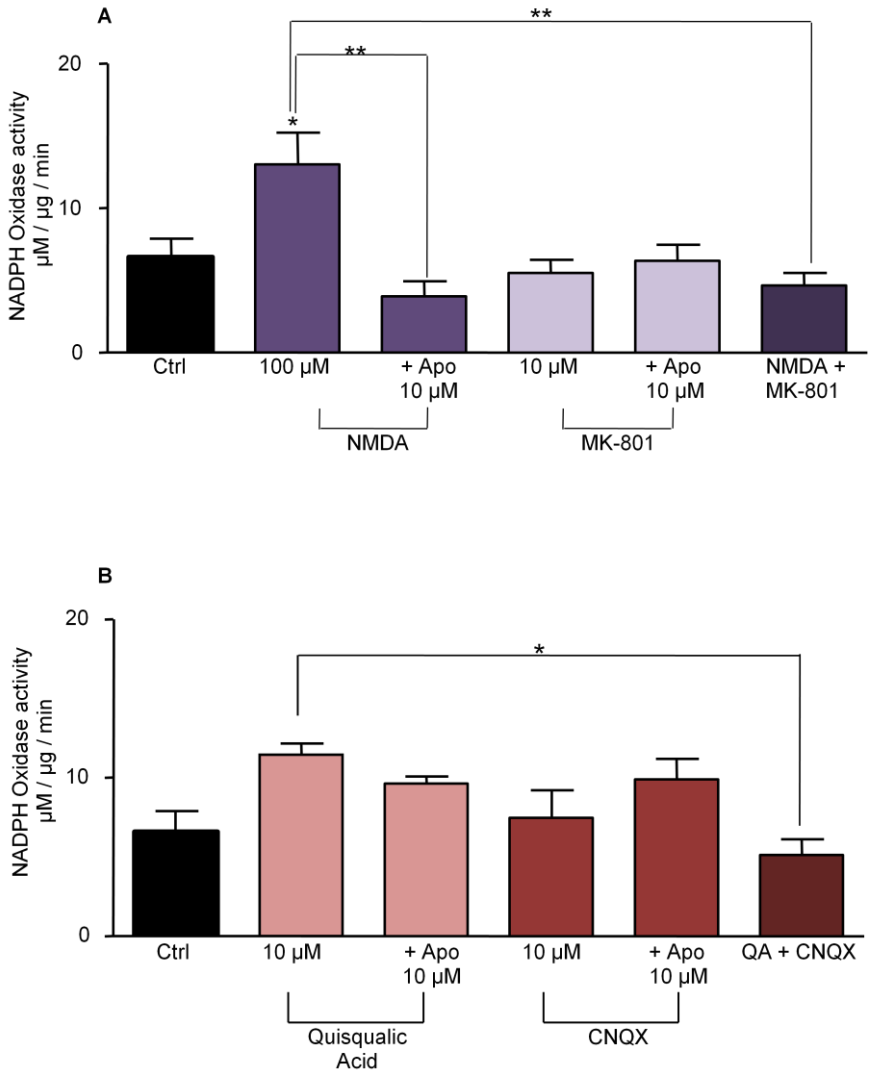

Figure 3.12 NADPH oxidase activity assay following modulation of the NMDA (A) and AMPA receptors (B). *BV2 microglia were treated with NMDA (100 µM) or MK-801 (10 µM) in the presence or absence of apocynin (10 µM) or in combination for 4 h. BV2 microglia were also treated with the AMPA receptor agonist QA (10 µM) or the antagonist CNQX (10 µM) in the presence or absence of apocynin (10 µM) or in combination for 4 h. NADPH oxidase activity was measured following 4 h incubation, and data was normalized to protein content. Data were analysed using a one way ANOVA with Tukey post hoc analysis comparing all treatments to basal NADPH oxidase activity, and also comparing treatments in the presence and absence of apocynin. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

3.2.2.3 Modulation of GABA receptors induces superoxide production in microglia

Following the finding that treatment of microglia with GABA induced superoxide production in an NADPH oxidase dependent manner, it was important to determine which GABA receptor was implicated in this superoxide production. Microglia express functional $GABA_B$ receptors (Charles et al. 2003), and treatment of microglia with the $GABA_B$ receptor agonist promotes an inward rectifying K+ current (Kuhn et al. 2004). Microglia respond to the $GABA_A$ receptor agonist muscimol, and receptor subunit expression is up-regulated on glioma cells co-cultured with neurons (Synowitz et al. 2001). These data therefore suggest that microglia may express the $GABA_A$ receptor under certain pathological conditions.

Superoxide production in response to the $GABA_A$ receptor agonist muscimol, or the $GABA_B$ receptor agonist SKF-97541 was investigated first using the NBT assay in BV2 microglia (Fig. 3.13A). A dose dependency analysis of each agonist was initally performed, using muscimol at 5 µM, 50 µM or 500 µM (Kuhn et al. 2004), and SKF-97541 at 1 µM, 10 µM or 100 µM (Synowitz et al. 2001). Treatment with the $GABA_A$ receptor agonist muscimol induced a significant increase in NBT reduction when used at 50 µM when compared with untreated controls, whereas treatment with the $GABA_B$ receptor agonist SKF-97541 did not significantly increase NBT reduction when each concentration was compared with untreated controls. To determine whether superoxide production induced as a consequence of microglial $GABA_A$ receptor activation was due to NADPH oxidase activation, BV2 microglia were co-treated with muscimol and apocynin (Fig. 3.13B). The significant increase in muscimol induced NBT reduction was attenuated by co-treatment of cells with the NADPH oxidase inhibitor, suggesting that activation of the $GABA_A$ receptor induces superoxide production in an NADPH oxidase dependent manner. It was important to determine whether these findings could be replicated in primary microglia (Fig. 3.13C, D). Primary microglia were treated with muscimol (50 µM) in the presence or absence of apocynin, or SKF-97541

(10 µM) in the presence or absence of apocynin for 24 h before superoxide production was assessed by dHEth fluorescence (Fig. 3.13D). Treatment with muscimol (50 µM) induced an increase in superoxide production that could be inhibited by co-treatment of cells with apocynin (Fig. 3.13C), in line with the NBT analysis. There was no change in superoxide production upon treatment of primary microglia with the $GABA_B$ receptor agonist SKF-97541, also in line with NBT data.

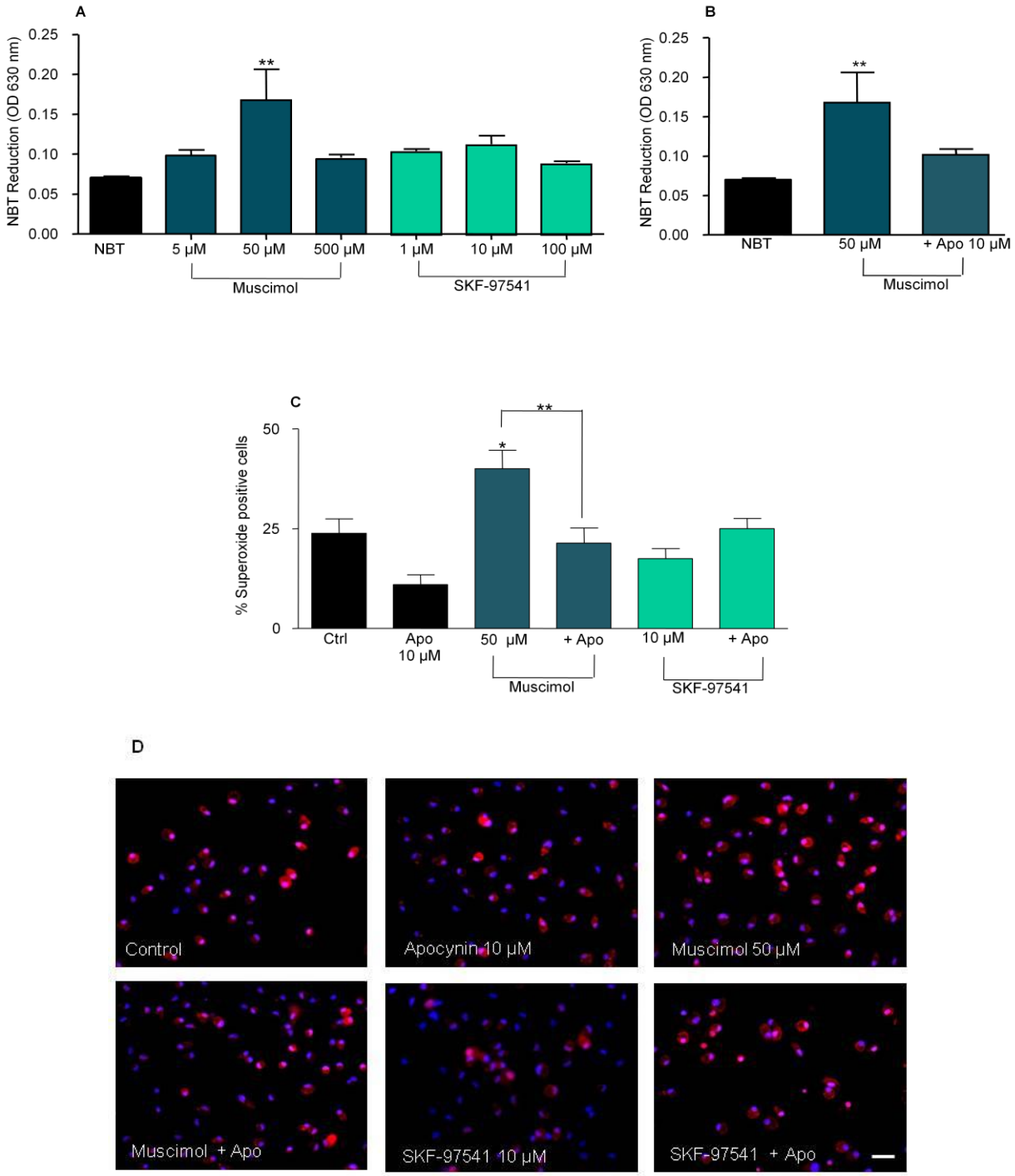

Figure 3.13 Analysis of superoxide production in BV2 and primary microglia following modulation of the $GABA_A$ and $GABA_B$ receptors. *A dose dependency analysis of $GABA_A$ and $GABA_B$ modulators was performed* ***(A).*** *BV2 microglia were treated with muscimol (5 µM, 50 µM or 500 µM) or SKF-97541 (1 µM, 10 µM or 100 µM) and NBT reduction was measured* ***(A).*** *BV2 microglia were then treated with muscimol (50 µM) in the presence and absence of apocynin (10 µM)* ***(B)*** *and NBT reduction was measured, to attribute superoxide production to the NADPH oxidase. The study was replicated in primary microglia using fluorescence imaging of dHEth* ***(C, D)*** *in which microglia were treated with muscimol (50 µM) or SKF-97541 (10 µM) in the presence or absence of apocynin (10 µM) and cells were counted to give the percent superoxide positive microglia. All data were analyzed using a One Way ANOVA and Tukey post-hoc analysis. *p<0.05, **p<0.01, ***p<0.001. All data are n=3. Scale bar 20 µm.*

It was important to quantify the muscimol induced superoxide production in microglia using flow cytometry (Fig. 3.14A, B) and HPLC (Fig. 3.14C). BV2 microglia were treated with muscimol (50 µM) in the presence or absence of apocynin (10 µM) for 24 h for both analyses. The flow cytometry data (Fig. 3.14 A, B) show a significant increase in the mean dHEth fluorescence intensity in cells treated with muscimol when compared with control untreated cells, which could be attenuated by co-treatment with apocynin, suggesting that activation of the $GABA_A$ receptor induced superoxide production in an NADPH oxidase dependent manner. The increase in dHEth fluorescence intensity was also replicated in the HPLC analysis, in which a significant increase 2-OH-E^+ production following muscimol treatment in comparison with untreated cells was observed, which was significantly reduced upon co-treatment with apocynin (Fig. 3.14C). Muscimol also enhanced Eth production in comparison with control cells, however, this was not attenuated by co-treatment with apocynin, suggesting that Eth production may be a result of muscimol induced ROS production through activation of other systems or other NADPH oxidase isoforms. As muscimol significantly elevated 2-OH-E^+ levels when compared with Eth levels, it could be suggested that modulation of the $GABA_A$ receptor predominantly acts on the NADPH oxidase to induce superoxide production.

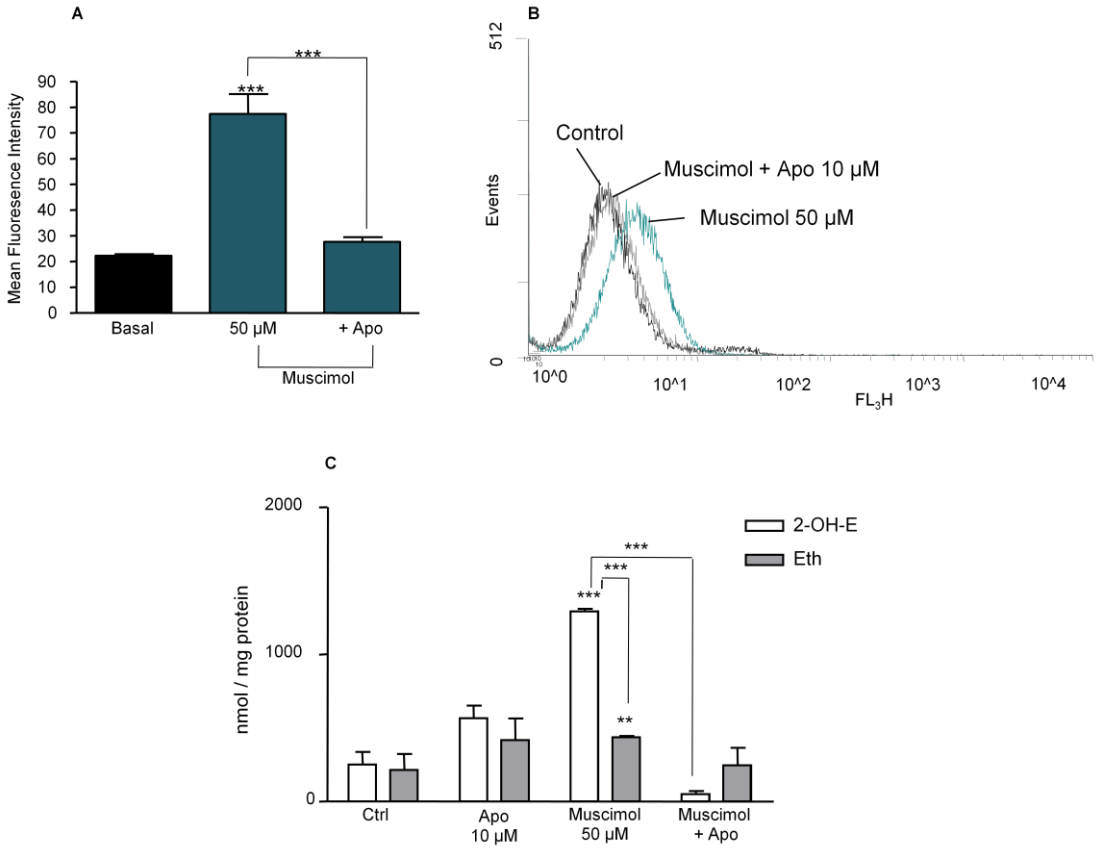

Figure 3.14. Quantitative analysis of $GABA_A$ induced superoxide production by flow cytometry of dHEth fluorescence and HPLC analysis of dHEth oxidation products. *BV2 microglia were treated with muscimol (50 µM) in the presence or absence of apocynin (10 µM) for 24 . For both flow cytometry and HPLC, cells were incubated with dHEth for 30 min.* ***(A)*** *shows the mean fluorescence intensity of dHEth by flow cytometry with a representative histogram shown in* ***(B).*** *Data were analysed by one way ANOVA and Tukey post-hoc analysis.* ***(C)*** *shows HPLC analysis of 2-OH-E and Eth production, and data were analysed by Student's T-Test and by two way ANOVA to compare 2-OH-E levels with Eth production. **p<0.01, ***p<0.001. All data are n=3.*

To confirm that muscimol induced superoxide production in microglia was a result of NADPH oxidase activation, an activity assay was performed (Fig. 3.15). BV2 microglia were treated with mucsimol (50 µM) in the presence or absence of apocynin (10 µM) for 4 h before NADPH oxidase activity was measured. The data confirmed that activation of the $GABA_A$ receptor with muscimol significantly increased NADPH oxidase activity when compared with control levels, and that this activity could be attenuated by co-treatment with apocynin (Fig. 3.15).

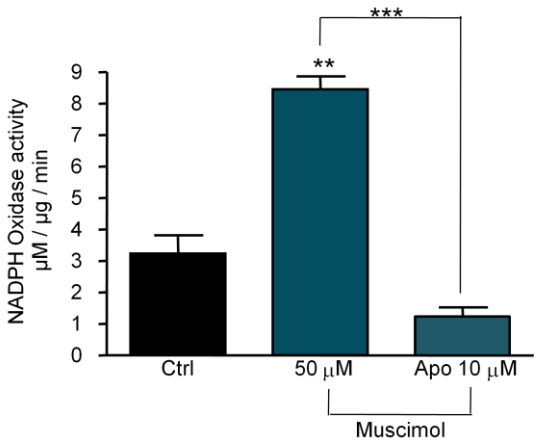

Figure 3.15 NADPH oxidase activity after treatment of BV2 microglia with muscimol. *BV2 microglia were treated with muscimol (50 µM) in the presence or absence of apocynin (10 µM) for 4 h. NADPH oxidase activity was measured and data was normalized to protein concentration. Data were analysed using a one way ANOVA with Tukey post-hoc analysis. **p<0.01, ***p<0.001. Data are n=3.*

Modulation of the microglial $GABA_A$ receptor therefore promotes superoxide production through activation of the NADPH oxidase. However, as the expression of the $GABA_A$ receptor in primary microglia is controversial, it was important to investigate whether primary microglia isolated from the rat brain expressed the appropriate subunits required for the formation of a functional $GABA_A$ receptor, and also to investigate whether modulation of the $GABA_A$ receptor by co-treatment with an agonist and an antagonist could attenuate superoxide production in primary microglia. Functional $GABA_A$ receptors are composed of tetramers of alpha, beta and gamma subunits (α2β2γ1). There are six alpha subunit isoforms, three beta and three gamma subunit isoforms which the $GABA_A$ receptor may be composed of. RT-PCR was used to determine whether primary microglia express the necessary subunits to form functional $GABA_A$ receptors (Fig. 3.16A). To determine whether the expression of these subunits changed depending on the activation state of microglia, cells were either left untreated, or were treated with the known microglial activator LPS (1 μg/ml). Untreated microglia express α2, α3 and α6 subunits, as well as β2 and γ3 subunits, suggesting that resting microglia express subunits that could form functional $GABA_A$ receptors (Fig. 3.16A). Expression of the α6 and β2 subunits were increased following LPS treatment of microglia (Fig. 3.16A), suggesting that functional $GABA_A$ receptors are more highly expressed on activated microglia, however, as expression of the γ subunits were not elevated, further functional analyses would be required to determine whether $GABA_A$ receptor activation was enhanced on reactive microglia.

It was also important to determine whether modulation of the $GABA_A$ receptor by co-treatment with muscimol and the $GABA_A$ receptor antagonists picrotoxin (Ptx 100 μM) or the highly selective $GABA_A$ receptor antagonist SR-95531 (10 μM) could inhibit superoxide production, to determine whether superoxide production was a direct result of activation of the $GABA_A$ receptor and not a consequence of an off target effect, such as activation of the

benzodiazepine receptor. Treatment of microglia with muscimol and Ptx or muscimol and SR95531 significantly decreased superoxide production induced by muscimol treatment alone (Fig. 3.16B), therefore $GABA_A$ receptor activation induces superoxide production. Furthermore, treatment with GABA (100 μM) as shown previously, also acts through the $GABA_A$ receptor, as co-treatment with GABA and Ptx or SR95531 significantly reduced GABA induced superoxide production in primary microglia (Fig. 3.16B).

Together, these data show that microglia express all subunits necessary for the formation of a functional $GABA_A$ receptor and that activation of the $GABA_A$ receptor with muscimol induces superoxide production through NADPH oxidase activation.

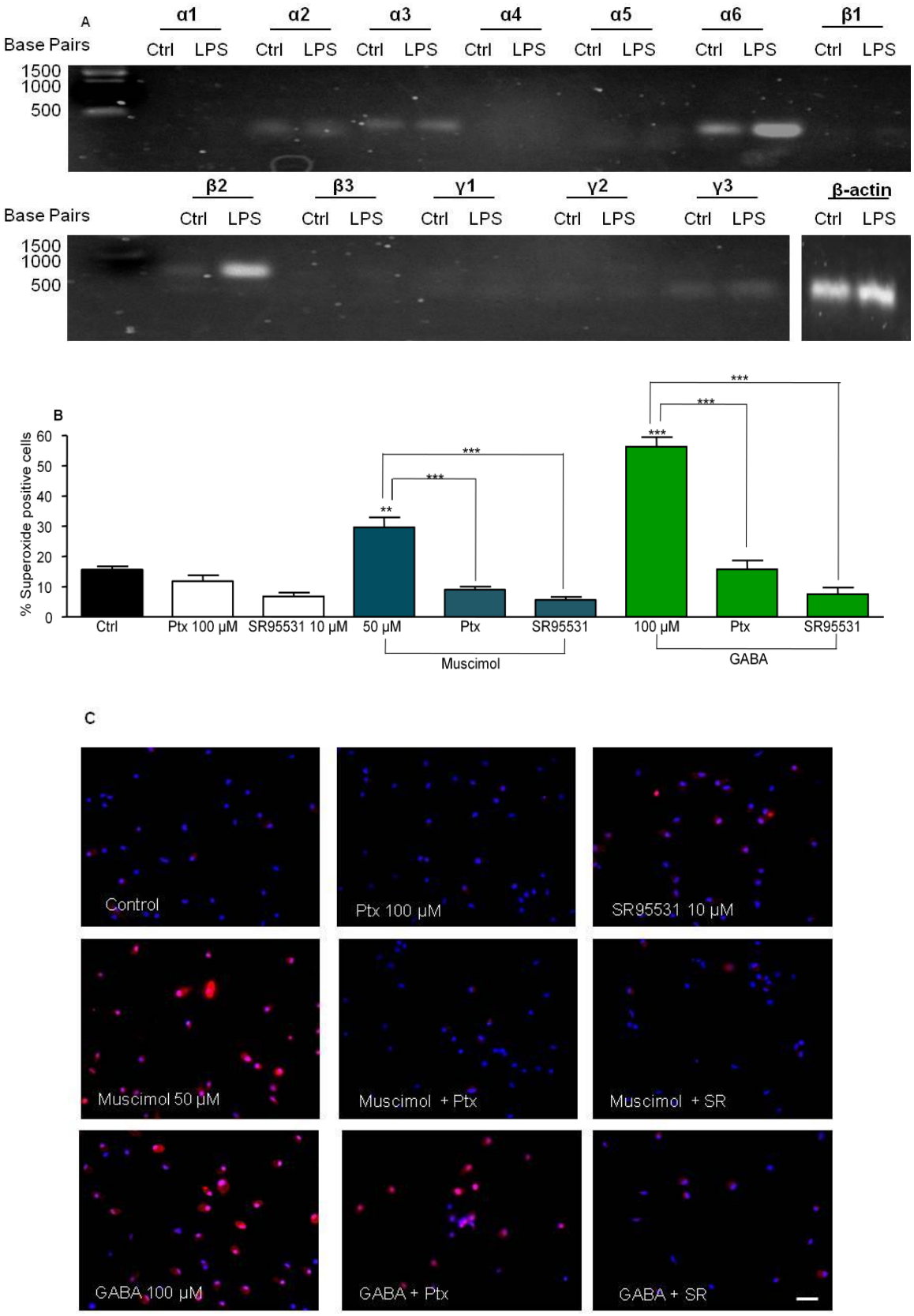

Figure 3.16 Legend overleaf

Figure 3.16 Analysis of $GABA_A$ receptor subunit expression (A) and modulation of superoxide production following co-treatment of microglia with $GABA_A$ receptor agonists and antagonists (B, C). *Primary microglia were either left untreated or were treated with LPS (1 µg/ml) for 24 h. Microglia were then subjected to RT-PCR for analysis of $GABA_A$ receptor subunit expression* ***(A).*** *PCR was performed using primers for six alpha subunits, and the three beta and three gamma subunits, and products were resolved on a 1% agarose gel. The experiment was performed in triplicate, and the gel presented here is representative. Primary microglia were then treated with the specific $GABA_A$ receptor antagonists Ptx (100 µM) or SR95531 (10 µM) plus muscimol (50 µM) or GABA (100 µM). Microglia were analysed for superoxide production by dHEth fluorescence imaging and microglia were counted to show the number of superoxide positive cells* ***(B, C)****. Data were analysed using a one way ANOVA and Tukey post-hoc analysis. **p<0.01, ***p<0.001. All data are n=3. Scale bar 20 µm.*

3.2.2.4 Modulation of the metabotropic purinergic receptors induces superoxide production in microglia

BzATP induced superoxide production in primary microglia, and is strong activator of the NADPH oxidase through its activation of the P2X7 receptor (Parvathenani et al 2003). As it is known that modulation of the ionotropic purinergic receptors can induce superoxide production, the effects of modulation of the metabotropic purinergic (P2Y) receptors on microglial superoxide production through NADPH oxidase activation were investigated. Microglia express the P2Y1, 2/4, 6, 7, 12 and 14 receptors, and expression of these receptors is modulated by the activation state of microglia (Bianco et al. 2005). Furthermore, P2Y receptor activation affects microglial motility, chemotaxis and phagocytosis (Pocock & Kettenmann 2007), suggesting that activation of these receptors has important consequences for microglial reactivity.

Here, superoxide production following P2Y receptor activation was first investigated using the NBT assay (Fig. 3.17A). The P2Y2/4 receptor was activated using UTPγS and a dose dependency analysis was performed in which BV2 microglia were treated with 1 μM, 10 μM or 100 μM UTPγS for 4 h. The optimal concentration required to induce a significant increase in NBT reduction was shown to be 100 μM (Fig. 3.17A), which induces an inward K^+ current in microglia (Bianco et al. 2005). The P2Y1 receptor was activated by MRS-2365, and a dose dependency NBT analysis was also performed in which BV2 microglia were treated with 10 nM, 100 nM or 1 μM (Fig. 3.17A), however, activation of the P2Y1 receptor did not significantly increase NBT reduction at any of the concentrations tested. Following the finding that UTPγS (100 μM) induced NBT reduction, it was important to determine whether this was dependent on NADPH oxidase activation. BV2 microglia were treated with UTPγS in the presence or absence of apocynin (10 μM) for 4 h and NBT reduction was assessed (Fig. 3.17B). Superoxide production induced by UTPγS was attenuated by inhibition

of the NADPH oxidase; therefore microglial P2Y2/4 receptor activation induces NADPH oxidase activity. It was important to determine whether superoxide production through activation of the P2Y2/4 receptor could be induced in primary microglia, therefore dHEth fluorescence imaging was performed using primary microglia treated with UTPγS (100 μM) in the presence or absence of apocynin (10 μM), and activation of the microglial P2Y1 receptor with MRS-2365 (100 nM) was also performed in the presence or absence of apocynin (10 μM) (Fig. 3.17C, D). Primary microglia were treated for 24 h before imaging of dHEth fluorescence. UTPγS significantly increased the percentage of superoxide positive microglia when compared with control untreated microglia, which was significantly reduced upon co-treatment with apocynin, whilst treatment with MRS-2365 had no effect on superoxide production in primary microglia (Fig. 3.17 C,D). These data therefore show that modulation of the P2Y2/4 receptor induces superoxide production in an NADPH oxidase dependent manner.

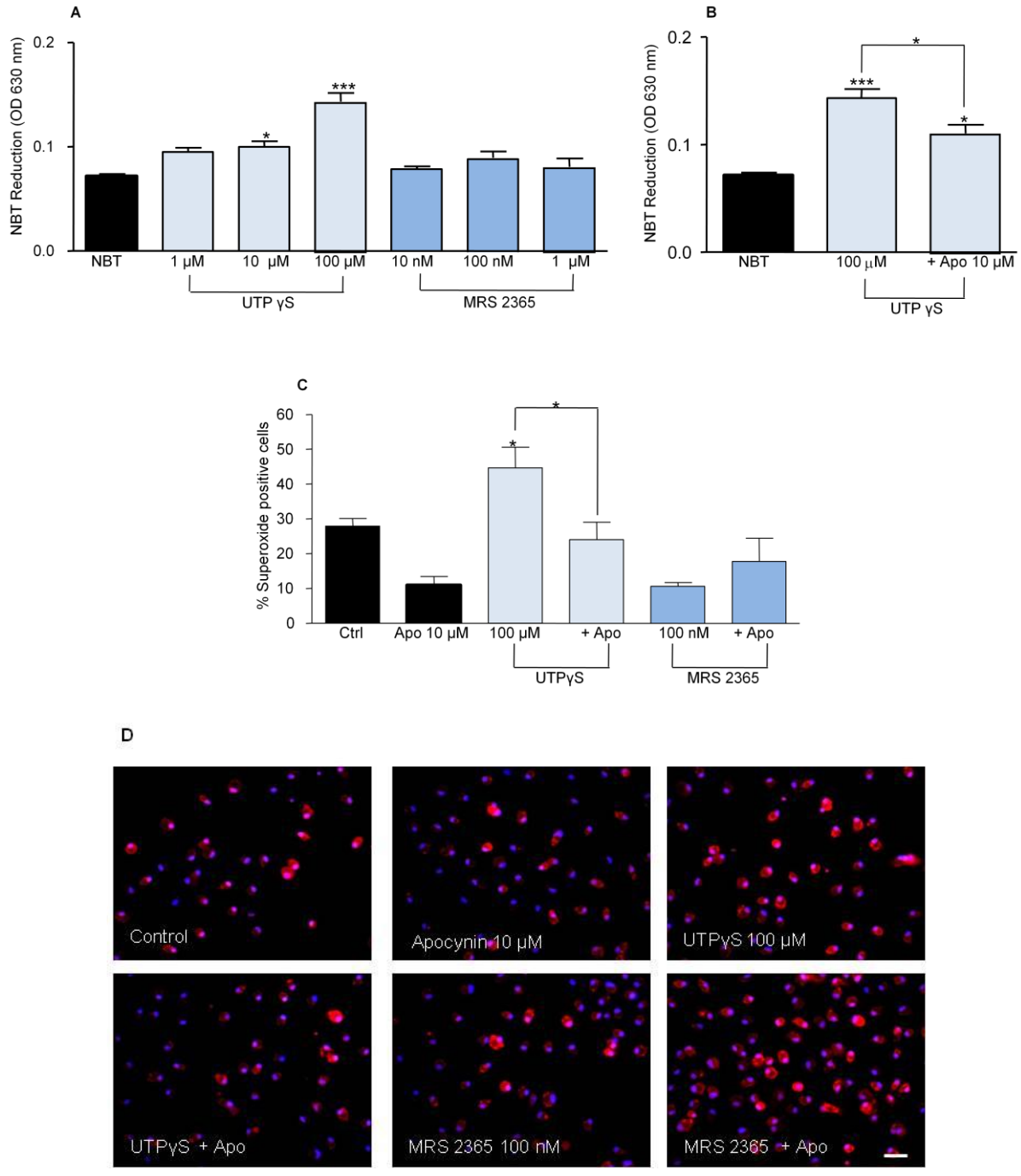

Figure 3.17 Analysis of superoxide production in BV2 and primary microglia after modulation of the P2Y2/4 and P2Y1 receptors. *BV2 microglia were treated with UTPγS (1 µM, 10 µM or 100 µM) or MRS-2365 (1 nM, 10 nM or 100 nM), for 4 h and were assessed for superoxide production by NBT reduction* ***(A).*** *BV2 microglia were then assessed for superoxide production following treatment with UTPγS (100 µM) in the presence or absence of apocynin (10 µM) for 4 h, using the NBT assay* ***(B).*** *The study was replicated in primary microglia* ***(C, D)****, in which superoxide production was assessed by dHEth fluorescence. Microglia were treated with UTPγS (100 µM) or MRS-2365 (100 nM) in the presence or absence of apocynin (10 µM), and superoxide production was assessed after 24 h incubation by counting the dHEth fluorescent cells* ***(C)****. All analysis was performed using a one way ANOVA with Tukey post-hoc analysis. *p<0.05, ***p<0.001. All data are n=3. Scale bar 20 µm.*

Quantitative analysis of dHEth fluorescence following treatment of BV2 microglia with UTPγS was performed by flow cytometry (Fig. 3.18A, B) and HPLC (Fig. 3.18C). The flow cytometry data showed that treatment of BV2 microglia with UTPγS (100 μM) for 24 h significantly increased mean dHEth fluorescence intensity when compared with control untreated cells, which could be significantly reduced by co-treatment with apocynin (10 μM) (Fig. 3.18A). HPLC analysis (Fig. 3.18C) agreed with these findings, showing that treatment with UTPγS significantly elevated the production of the dHEth superoxide specific oxidation product, 2-OH-E^+, when compared with untreated controls, which was attenuated by co-treatment with apocynin. Furthermore, 2-OH-E^+ production by UTPγS was significantly elevated in comparison to Eth production, suggesting that UTPγS preferentially activates the NADPH oxidase to induce superoxide production.

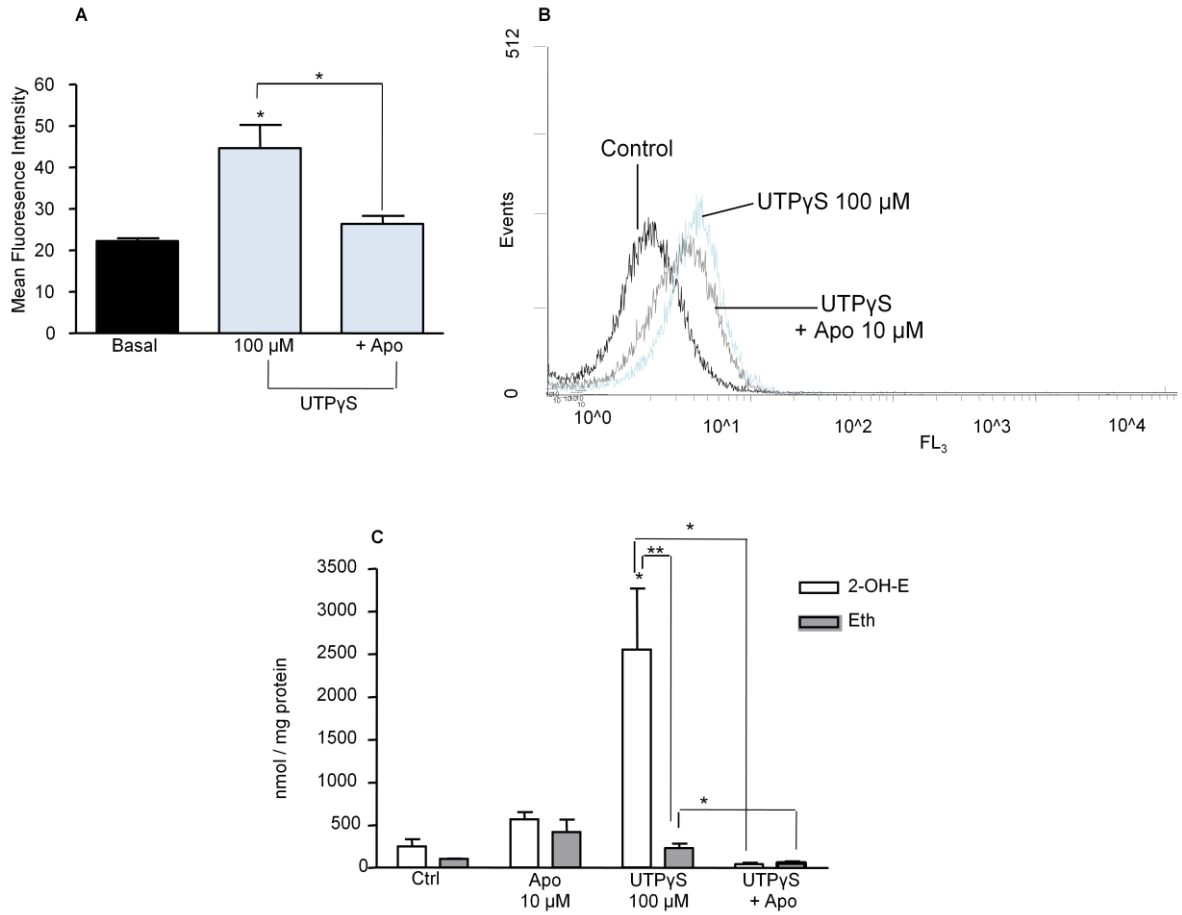

Figure 3.18 Quantitative analysis of P2Y2/4 receptor induced dHEth fluorescence by flow cytometry and of dHEth oxidation products by HPLC. *Flow cytometry* ***(A)*** *was used to quantify dHEth fluorescence after treatment of BV2 microglia with UTPγS (100 µM) for 24 h in the presence or absence of apocynin (10 µM). The mean fluorescence intensity is shown* ***(A)*** *along with a representative histogram* ***(B).*** *Data were analysed by one way ANOVA and Tukey post-hoc analysis. HPLC analysis was performed to determine the production of the dHEth oxidation products 2-OH-E and Eth after treatment of BV2 microglia with UTPγS (100 µM) in the presence or absence of apocynin (10 µM) for 24 h* ***(C).*** *Data were analyzed by Student's T-Test and two way ANOVA for comparisons between 2-OH-E and Eth production. *p<0.05, **p<0.01. All data are n=3*

It was important to confirm that superoxide production as a result of UTPγS treatment of microglia was a consequence of increased NADPH oxidase activity, therefore an NADPH oxidase activity assay was performed in which BV2 microglia were treated with UTPγS (100 μM) in the presence or absence of apocynin (10 μM) for 4 h. Enzymatic activity was measured and shown in Fig. 3.19. Treatment with UTPγS significantly increased NADPH oxidase activity when compared with control untreated cells, and this activity was attenuated by co-treatment of cells with apocynin, therefore indicating that superoxide production from P2Y2/4 receptor activation is a consequence of increased NADPH oxidase activity.

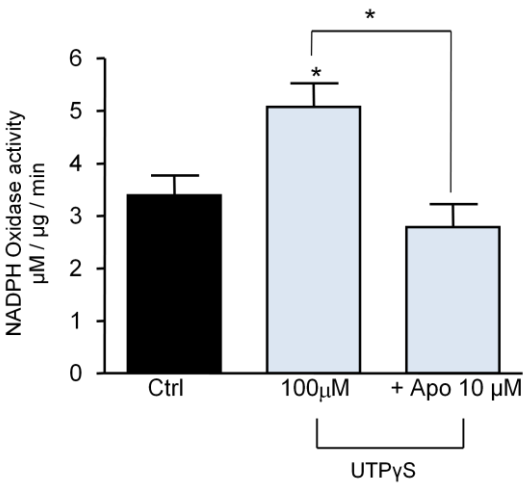

Figure 3.19 NADPH oxidase activity after treatment of BV2 microglia with UTPγS. *BV2 microglia were treated with UTPγS (100 µM) for 4 h in the presence or absence of apocynin (10 µM) and NADPH oxidase activity was measured. The data were analysed using a one way ANOVA with Tukey post-hoc analysis. *p<0.05. Data are n=3.*

3.3 Discussion

The data presented in this chapter show that NADPH oxidase derived superoxide production in microglia can be induced by the neurotransmitters glutamate, GABA or BzATP, and agonists or antagonists of the corresponding neurotransmitter receptors. Four different methods were used to assess superoxide production, and NADPH oxidase activity was also analysed. The data presented here are therefore a comprehensive analysis of superoxide production in microglia as a consequence of neurotransmitter and receptor modulation.

3.3.1 Neurotransmitters modulate superoxide production in microglia

Treatment with the neurotransmitters glutamate, GABA or BzATP significantly increased superoxide production when compared with control un-treated cells. Glutamate significantly elevated superoxide production when used at 1 μM, which is within the physiological concentration range of 1-4 μM found at non-synaptic sites (Baker et al. 2002; Nyitrai et al. 2006). However, microdialysis has shown that glutamate is present at lower concentrations in the CNS, with levels of 25 nM reported in resting hippocampal slices (Herman & Jahr 2007). It has however been shown that physiological stimulation of glutamate receptors occurs within the micromolar range, thereby indicating that the glutamate concentration used here may be physiological. Furthermore, the EC_{50} for the NMDA receptor is reported to be 2.3 μM (Patneau & Mayer 1990), and the EC_{50} for the group I mGluRs is between 3-13 μM, for mGluR3 is 4-5 μM and for the group III mGluRs is between 0.02 and 1000 μM (Conn & Pin, 1997), thereby suggesting that micromolar and therefore physiological levels of glutamate may promote the stimulation of microglial glutamate receptors. According to Herman & Jahr (2007) the concentration of glutamate used here may not lie within the basal range, and may be more representative of either physiological stimulation, or excitotoxic injury seen during ischaeimia and in AD, in which degenerating neurons and activated astrocytes release glutamate into the extracellular space (Vesce et al. 2007). Here, treatment

of BV2 microglia with lower levels of glutamate (100 nM) did not significantly elevate NBT reduction, suggesting activation of the microglial NADPH oxidase following exposure to glutamate may only occur under physiological stimulatory conditions or during pathology, rather than in response to basal glutamate levels.

GABA is present at extrasynaptic sites at a concentration of 2.9 µM (Lerma et al. 1986), whilst here, exposure of microglia to 100 µM GABA induced a more significant increase in NBT reduction, and subsequent superoxide production when compared with exposure to lower concentrations. Treatment of BV2 microglia with 1 µM GABA significantly elevated NBT reduction; however, as treatment with the higher concentration of 100 µM induced a more significant increase in NBT reduction, this was used in later experiments. These data do however suggest that microglia respond to physiological concentrations of GABA as well as higher concentrations, which may mimic the increase in GABA tone seen during AD pathology (DiFiglia 1990). GABA is released from neurons during AD (Marczynski 1998), and these elevated GABA levels exacerbate neuronal vulnerability (Erdö et al. 1991). It could be suggested that the GABA released in neurodegenerative processes could act on microglia to enhance reactivity and induce ROS production.

Whilst treatment of BV2 and primary microglia with GABA (100 µM) siginifcantly elevated superoxide production that could be inhibited by apocynin as determined by the NBT assay and by dHEth fluorescence microscopy, flow cytometry analysis showed that GABA induced dHEth fluoresenence could not be significantly attenuated upon co-treatment with apocynin, suggesting the involvement of another oxidase system or activation of other NADPH oxidase isoforms, such as Nox4, which are insensitive to apocynin (Harrigan et al. 2008). Furthermore, HPLC analysis showed that GABA treatment significantly increased Eth production, indicative of H_2O_2 generation (Zielonka et al. 2008), which was significantly reduced by apocynin, demonstrating that GABA induces ROS, rather than superoxide

production through activation of the NADPH oxidase. The NADPH oxidase activity assay showed that enzymatic activity was increased upon treatment with GABA, which could be attenuated by co-treatment with apocynin, supporting the findings that GABA induced ROS production was a consequence of NADPH oxidase activity, however this finding does not rule out the possibility that GABA may induce the activation of additional NADPH oxidase isoforms.

Superoxide production from the NADPH oxidase isoforms Nox1 and Nox4, shown to be expressed in microglia (Harrigan et al. 2008; Chéret et al. 2008), is more rapidly dismutased to H_2O_2 than superoxide produced from Nox2 (Dikalov et al. 2008; Lassègue & Griendling 2010), which suggests that GABA induces microglial Nox1 or Nox4 activation. The findings could however also suggest that exposure of BV2 microglia to GABA may enhance the enzymatic activity or expression of SOD, which converts superoxide to H_2O_2 (Vaziri et al. 2004). This has been demonstrated in an *in vivo* model of rat renal failure, in which administration of GABA to the kidney increased SOD expression, which protected against oxidative damage through the enhanced production of H_2O_2 rather than superoxide (Sasaki et al. 2006). There are no reports of this in the CNS; however it could be an interesting point for further investigation.

Treatment of BV2 and primary microglia with 250 µM BzATP significantly increased superoxide production in line with published findings (Parvathenani et al. 2003; Skaper et al. 2006). BzATP was used in place of ATP, which was readily hydrolysed in the culture media, and had no effect on microglial reactivity or the production of superoxide *in vitro* (Skaper et al. 2006), however, ATP is released from dying neurons as an activating signal for microglia, and therefore exerts an effect *in vivo* (North & Verkhratsky 2006). Whilst BzATP induced superoxide production in primary and BV2 microglia, it did not significantly elevate NADPH oxidase activity. These findings therefore suggest that treatment of BV2 microglia with

BzATP may induce superoxide production through NADPH oxidase activation, and also by another superoxide generating system.

Superoxide production following treatment of BV2 microglia with BzATP was observed by HPLC analysis, showing a significant increase in 2-OH-E^+, which was attenuated by apocynin. However, Eth production was also elevated, which could not be attenuated by co-treatment with apocynin, suggesting a low level of activity of another H_2O_2 generating system, or enhanced activation of the mitochondrial respiratory chain, leading to increased release of H_2O_2 (Rigoulet et al. 2011). There are reports that activation of microglia with Aβ promotes ATP release which activates the NADPH oxidase in an autocrine manner, elevating ROS production rather than superoxide specifically (Moon et al. 2008), which supports the elevated Eth seen here by HPLC. Furthermore, activation of the microglial P2X7 receptor with BzATP promotes TNFα production (Suzuki et al. 2004) which elevates ROS generation from the mitochondria (Goossens et al. 1999). It could therefore be suggested that whilst BzATP induces superoxide production through the NADPH oxidase, the production of TNFα as a consequence of increased microglial reactivity following activation of the P2X7 receptor could increase H_2O_2 production through mitochondrial pathways (Morgan & Liu 2010). In addition, TNFα release from microglia following activation of the P2X7 receptor (Suzuki et al. 2004) can feed-back onto microglial TNF receptors (TNFR's) in an autocrine manner (Kuno et al. 2005), resulting in elevated ROS production through enhanced expression and activity of the NADPH oxidase (Mir et al. 2009) and also mitochondrial pathways (Suzuki et al. 2004). The contribution of TNFα induced ROS, produced as a consequence of P2X7 receptor activation of microglia could explain why here, increased superoxide production is observed in the assays detecting ROS production, but that the NADPH oxidase activity assay shows a non-significant increase in enzymatic activity. The NADPH oxidase does indeed

play a role in BzATP induced production of superoxide in microglia, however the levels may be enhanced by TNFα induced activation of the mitochondrial respiratory pathway.

3.3.2 Modulation of neurotransmitter receptors induces superoxide production in microglia

Modulation of microglial glutamate, GABA and purinergic receptors induced NADPH oxidase derived superoxide production. In line with published findings, the group I mGluR agonist DHPG did not induce NADPH oxidase derived superoxide production in BV2 microglia (Loane et al. 2009). However, treatment of BV2 microglia with CDPPB (an mGluR5 specific agonist) increased NBT reduction. The agonist CDPPB may induce superoxide production through the activation of other oxidase systems. Activation of neuronal mGluR5 promotes mitochondrial ROS production, which elevates neuronal excitability (Li et al. 2011), suggesting that in BV2 microglia, mGluR5 activation may promote mitochondrial ROS production, whilst antagonism of the mGluR5 mediates NADPH oxidase derived superoxide production.

Antagonism of the group I mGluRs (mGluR5) with MTEP significantly increased superoxide production in an NADPH oxidase dependent manner. Activation of mGluR5 promotes PLC signalling (Mao &Wang 2002) which can attenuate NADPH oxidase activation in leukocytes (Traynor et al. 1993). Furthermore, inhibition of the astrocytic group I mGluRs can down-regulate PLC signalling, which correlates with reactive gliosis (Floyd et al. 2004), of which ROS production is a hallmark (Jekabsone et al. 2006), and agrees with the data presented here. In addition, neuronal group I mGluR activation inhibits glutamate release (Wiśniewski & Car 2002; Zhang et al. 2002), and treatment of astrocytes with the mGluR group I agonist ACPD decreased astrocytic glutamate release (Ye & Sontheimer 1999). Glutamate released from neurons can facilitate microglial activation (Rogove & Tsirka 1998) and NADPH oxidase activity (Harrigan et al. 2008). It could be suggested that in microglia, activation of

the group I mGluRs inhibits glutamate release, thereby inhibiting an autocrine feedback of glutamate onto other microglial glutamate receptors, preventing NADPH oxidase activation. In contrast, antagonists of the group I mGluRs may induce glutamate release from microglia, which could feedback onto mGluRs or iGluRs, inducing superoxide production through activation of these receptors in addition to NADPH oxidase activation through the direct effect of inhibition of the group I mGluR (Fig. 3.20).

Activation of mGluR3 with NAAG induced microglial superoxide production in an NADPH oxidase dependent manner. Activation of the neuronal mGluR3 elevates PKC activity (Tyszkiewicz et al. 2004), which promotes NADPH oxidase activation through phosphorylation of the cytoplasmic subunits (Yamamori et al. 2000). Furthermore, mGluR3 mediated PKC activation promotes NMDA receptor activation (Tyszkiewicz et al. 2004), which enhances NADPH oxidase activity (Zhang et al. 2010), suggesting that mGluR3 activation may enhance NADPH oxidase activity directly through PKC signalling and indirectly through NMDA receptor activation.

Treatment of BV2 and primary microglia with NAAG induced superoxide production that could be detected by the NBT assay, dHEth fluorescence microscopy, and flow cytometry, however, HPLC analysis did not show a significant increase in 2-OH-E^+ or Eth when compared with control untreated cells. This could suggest that multiple NADPH oxidase isoforms are induced by mGluR3 activation. The inability of NAAG to significantly elevate 2-OH-E^+ or Eth levels could however be a result of increased antioxidant production. Activation of mGluR3 in dorsal root neurons co-cultured with Schwann cells induced an increase in glutathione, which reduced ROS production (Berent-Spillson & Russell 2007). It could therefore be suggested that in microglia, NAAG elevates ROS production through modulation of the NADPH oxidase, however NAAG may also increase the production of free

radical scavengers, which would reduce the concentration of detectable ROS (Fig. 3.20), leading to the non-significant effect observed by HPLC analysis.

Treatment of BV2 and primary microglia with the group III activator L-AP4 significantly increased NADPH oxidase derived superoxide production. Lymphocytes express functional group III mGluRs, and treatment with L-AP4 elevates ROS production (Boldyrev et al. 2004), in support of the findings here. Furthermore, group III mGluR induced ROS production in lymphocytes facilitates the communication between the immune and nervous systems (Boldyrev et al. 2005), which is supported by findings that ROS production in dendritic cells can regulate the T-cell response (Olofsson et al. 2003).The predominant dHEth oxididation product observed by HPLC was 2-OH-E^+, suggesting that superoxide is specifically induced by activation of the group III mGluRs, and NADPH oxidase activity was increased by treatment with this agonist. Activation of group III mGluRs in the rat entorhinal cortex promotes glutamate release and PKC signalling (Evans et al. 2001), which promotes NADPH oxidase activation through p47phox phosphorylation (Raad et al. 2009), suggesting a mechanism for L-AP4 mediated NADPH oxidase activation. In addition, activation of the neuronal group III mGluRs mediates PI3-K signalling (Iacovelli et al. 2002), which mediates NADPH oxidase activation through phosphorylation of the cytoplasmic p40phox (Ellson et al. 2006). It could therefore be suggested that a combination of effects such as enhanced PKC or PI3-K activity and excess glutamate release could contribute to the NADPH oxidase derived superoxide production in microglia following activation of the group III mGluRs seen here (Fig. 3.20).

Modulation of the microglial iGluRs induced superoxide production in an NADPH oxidase dependent manner. Treatment of microglia with NMDA significantly elevated superoxide production which could be inhibited by apocynin. Furthermore, co-treatment of microglia with NMDA and the NMDA receptor antagonist MK-801 inhibited superoxide production,

suggesting that direct modulation of the NMDA receptor induced NADPH oxidase mediated superoxide production. These findings are supported by *in vivo* studies demonstrating that direct activation of the NMDA receptor mediates NADPH oxidase derived superoxide production (Zhang et al. 2010). HPLC analysis of dHEth oxidation following treatment of BV2 microglia with NMDA showed a significant increase in the superoxide specific product 2-OH-E^+; however, Eth was also elevated, suggesting NMDA also induced H_2O_2 production. In support of this finding, NMDA treatment of cortical neurons promotes Nox4 mediated H_2O_2 production (Ha et al. 2010). Induction of ROS and superoxide production in microglia could therefore originate from the activation of more than one Nox isoform, which could explain the almost equal production of 2-OH-E^+ and Eth.

The findings that NMDA mediated superoxide production could be attenuated by co-treatment with the antagonist MK-801 lends support to findings that microglia *in vivo* express functional NMDA receptors, and that activation of these NMDA receptors mediates NO production, suggesting that modulation of the microglial NMDA receptor induces the production of free radicals (Murugan et al. 2011). Whilst the findings reported here that modulation of the microglial NMDA receptor induces superoxide in an NADPH oxidase dependent manner are novel, it has been reported that in neurons, modulation of the NMDA receptor mediates NADPH oxidase derived superoxide production, as inhibition of NADPH synthesis prevented NMDA receptor mediated superoxide production (Brennan et al. 2009), in line with the findings reported here. *In vivo* models have shown that application of NMDA to the mouse neocortex significantly elevated ROS production, which was not seen in Nox2 knock-out mice, suggesting that NMDA induced ROS production was a consequence of NADPH oxidase activation (Girouard et al. 2009). Activation of the neuronal NMDA receptor induces PKCζ activity (Koponen et al. 2003), which mediates NADPH oxidase

activation by p47phox phosphorylation (Leverence et al. 2011), and could be implicated in microglial NMDA induced NADPH oxidase activation (Fig. 3.20).

Treatment of BV2 and primary microglia with the NMDA receptor antagonist MK-801 also induced superoxide production, however this could not be attenuated by co-treatment with apocynin. Blockade of the mouse NMDA receptor with ketamine *in vivo* initially elevates NADPH oxidase dependent ROS production, however prolonged exposure down-regulates Nox2 activity, and induces glutamate release (Sorce et al. 2010). The increase in superoxide production observed following NMDA receptor antagonism shown here could be a combinatorial effect of NADPH oxidase dependent superoxide production as well as the production of superoxide from other apocynin insensitive Nox isoforms (such as Nox4). Furthermore, blockade of neuronal NMDA receptors with ketamine elevates IL-6 production which enhances inflammation, and is associated with increased ROS production (Behrens et al. 2008) through Nox4 activation (Li et al. 2009). The production of IL-6 from blockade of NMDA receptors could therefore contribute to the increase in ROS observed following treatment of BV2 microglia with MK-801 (Fig. 3.20).

Modulation of the microglial AMPA receptor did not affect superoxide production. Activation of the AMPA receptor with QA failed to induce superoxide production that could be detected by any method in either BV2 or primary microglia, in aggrement with findings in cortical neurons, in which activation of the AMPA receptor did not mediate H_2O_2 production (Ha et al. 2010). In hippocampal neurons, application of AMPA mediates superoxide production, however this increase was counterbalanced by elevated SOD activity, and AMPA induced superoxide production was only observed after SOD knock-down (Rego et al. 2003). Furthermore, activation of the AMPA receptor *in vivo* was shown to increase superoxide production, but also elevated SOD expression, and the effect could be reversed after co-treatment of rats with AMPA and the receptor antagonist CNQX (Radenović et al. 2005).

Any increase in superoxide production seen here may therefore be masked by an up-regulation of SOD. Increased superoxide production was observed following antagonism of the AMPA receptor with CNQX. This could not be inhibited with apocynin however, suggesting that the NADPH oxidase was not activated. Modulation of the neuronal AMPA receptor induces superoxide production from the mitochondria (Radenović et al. 2005). Furthermore, CNQX was shown to inhibit SOD production in hippocampal neurons (Radenović et al. 2005), suggesting that the increase in superoxide observed in microglia shown here could be a result of decreased SOD activity and increased mitochondrial ROS production.

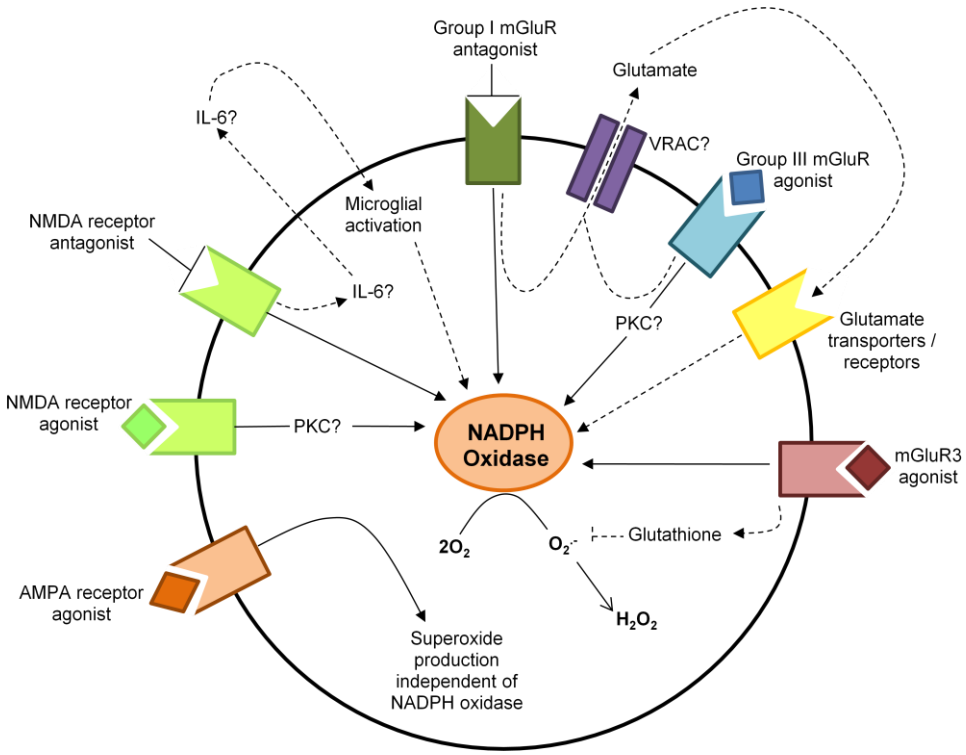

Figure 3.20 Possible mechanisms of NADPH oxidase activation following modulation of microglial glutamate receptors. *Microglia treated with the mGluR group I antagonist increased superoxide production. This could be direct, but also through the release of glutamate, which may feed back onto other glutamate receptors or transporters. Activation of the group III mGluR's induced superoxide production, possibly through PKC activation, however glutamate has also been shown to be released from glial cells following modulation of this receptor, which could feedback onto microglia. Activation of mGluR3 induced superoxide production, however levels were low. It has been suggested that modulation of this receptor induces an increase in glutathione, which may reduce superoxide levels. Activation of the AMPA receptor did not induce superoxide production, whilst activation of the NMDA receptor could induce superoxide production which may be through modulation of PKC. Inhibition of NMDA receptors also induced superoxide production, which could be direct, or may be a result of production of IL-6 and subsequent microglial activation.*

Activation of the microglial $GABA_A$ receptor significantly increased superoxide production. There is some debate over the expression of functional $GABA_A$ receptors on microglia (Synowitz et al. 2001); however functional $GABA_A$ receptors have been identified on human glioma cells (Labrakakis et al. 1998), and more recently on human microglia (Lee et al. 2011). Here, a significant decrease in superoxide production was observed in primary microglia following co-treatment with the $GABA_A$ receptor agonist in the presence of the antagonists Ptx or SR95531 suggesting that direct activation of the $GABA_A$ receptor mediates superoxide production. Furthermore, primary microglia were shown to express the subunits required for the assembly of a functional $GABA_A$ receptor, and expression of these subunits was elevated upon microglial activation, in agreement with findings that microglia in gliomas express $GABA_A$ receptors, suggesting that increased microglial reactivity up-regulates $GABA_A$ receptor expression (Synowitz et al. 2001). The suggestion that activated microglia express functional $GABA_A$ receptors may indicate that the microglial cultures prepared here could be basally activated as a consequence of dissociation from other cell types (Tham et al. 2003), as untreated microglia expressed $GABA_A$ receptor subunits and responded to muscimol. It would be important to investigate this further using immunocytochemistry to determine whether microglia in co-culture with neurons or microglia in organotypic slice cultures expressed $GABA_A$ receptors. However, microglial cultures prepared using the method in this thesis express low levels of the activation marker ED1 (Morgan et al. 2004), and in support of the findings that resting microglia express subunits of the $GABA_A$ receptor, ramified human microglial cells have recently been shown to express protein and mRNA for the $GABA_A$ receptor, and modulation of these receptors with muscimol induces TNFα and IL-6 release (Lee et al. 2011). It could therefore be suggested that TNFα reseased following $GABA_A$ receptor activation could feedback onto microglial TNFR's to induce NADPH oxidase activation and subsequent superoxide production (Shen et al. 2004) (Fig. 3.21)

HPLC analysis of dHEth oxidation following treatment of BV2 microglia with muscimol significantly elevated both 2-OH-E^+ and Eth, suggesting that activation of the $GABA_A$ receptor induces ROS and superoxide production. However, 2-OH-E^+ production was significantly higher than Eth production, and could be attenuated by co-treatment with apocynin. The production of Eth corresponds with the GABA data, in which treatment with GABA alone significantly elevated Eth levels, suggesting that GABA may act through the $GABA_A$ receptor to induce superoxide production in an NADPH oxidase dependent manner. The production of Eth corresponds with Nox4 induction, and modulation of the $GABA_A$ receptor up-regulates Nox4 activation in endothelial cells (Tyagi et al. 2009). Furthermore, activation of the neuronal $GABA_A$ receptor mediates MAPK signalling (Obrietan et al. 2002), which mediates Nox4 activation and ROS production (Wagner et al. 2007).

There are no published reports regarding the effects of modulation of the $GABA_A$ receptor on superoxide production either in neuronal or glial cells. However, ROS production facilitates the binding of ligands to the $GABA_A$ receptor (Yoneda et al. 1985; Sah et al. 2002). These data show that pre-treatment of cells with xanthine to induce activation of the xanthine oxidase system, and subsequent superoxide production, increases the binding of muscimol to $GABA_A$ receptors, and elevates superoxide production (Yoneda et al. 1985). Furthermore, exposure of the rat hippocampus to H_2O_2 also increased muscimol binding to $GABA_A$ receptors (Sah et al. 2002). It could therefore be suggested that treatment of microglia with muscimol induces ROS production through Nox4, and that this ROS facilitates the binding of muscimol to the $GABA_A$ receptor, which may perpetuate ROS production through increased activation of the $GABA_A$ receptor (Fig. 3.21).

Modulation of the metabotropic purinergic (P2Y) receptors on microglia promoted superoxide production through NADPH oxidase activation. Treatment of microglia with the P2Y2/4 receptor agonist UTPγS significantly elevated superoxide production, which could be

inhibited by apocynin, suggesting that activation of this receptor induced NADPH oxidase activity. This was confirmed by the NADPH oxidase activity assay. The main oxidative species produced from treatment of BV2 microglia with UTPγS was superoxide, as demonstrated by HPLC analysis, in which there was a significant increase in 2-OH-E^+ production when compared with control untreated cells, and also when compared with the production of Eth. Modulation of the P2Y1 receptor did not significantly elevate superoxide production in BV2 or primary microglia. In rat alveolar macrophages, activation of the P2Y1 receptor with ADP promotes superoxide release through the respiratory burst (Gozal et al. 2001), as opposed to the production of intracellular superoxide measured here, suggesting that modulation of the microglial P2Y1 receptor may enhance the production of released superoxide, which could be investigated further.

The microglial P2Y receptors are implicated in microglial chemotaxis (Ohsawa et al. 2007), phagocytosis (Koizumi et al. 2007) and the movement of microglial fine processes (Haynes et al. 2006). Little however, is known about the function of the microglial P2Y2/4 receptor, other than it is expressed on microglia (Boucsein et al. 2003; Bianco et al. 2005), it's expression remains constant regardless of miroglial activation state, and receptor activation promotes an inward rectifying K^+ current, suggesting that activation of these receptors mediates microglial reactivity (Bianco et al. 2005). P2Y2/4 receptors have been linked to the stimulation of pathways involving p38-MAPK, and PKC isoforms, which are both implicated in NADPH oxidase activation (Guerra et al. 2007), although there are no published data regarding the direct effect of modulation of P2Y2/4 on the microglial NADPH oxidase activation to date. It has however been shown that treatment of human neutrophils with UTP can induce superoxide production (Kuhns et al. 1988), and can enhance the superoxide produced following priming of neutrophils with toxic moieties such as fMLP in an NADPH oxidase dependent manner (Seifert et al. 1989). Furthermore, modulation of the P2Y2/4

receptor with UTP promotes ROS production and mediates intracellular Ca^{2+} mobilisation in human eosinophils, and chelation of intracellular calcium inhibits ROS production (Ferrari et al. 2000). The findings that in immune cells modulation of the P2Y2/4 receptor induces superoxide production therefore lends support to the finding here that activation of the microglial P2Y2/4 receptor induces NADPH oxidase activity and superoxide production, which could have an important immune regulatory role. These data also suggest a role for intracellular calcium as a second messenger for NADPH oxidase activation, which could be an interesting route of further investigation, along with the activation of signalling pathways such as PKC and p38-MAPK (Fig. 3.21).

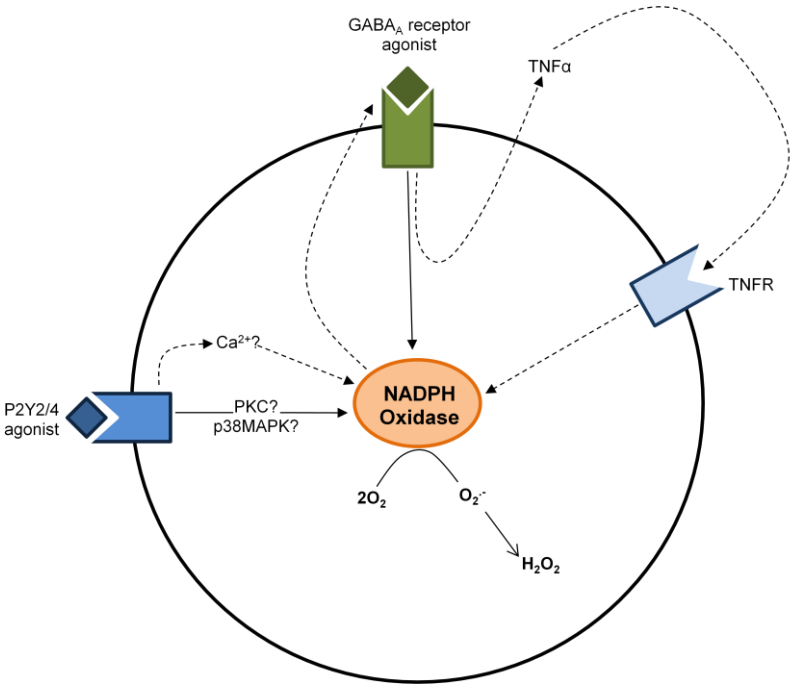

Figure 3.21. Possible mechanisms by which modulation of the GABA$_A$ and P2Y2/4 receptors may induce superoxide production in microglia. *Microglia treated with the GABA$_A$ agonist muscimol induced superoxide production, which may occur through direct mechanisms, but could also induce the release of TNFα, which may feedback onto TNFR's to induce activation of the NADPH oxidase. Activation of the P2Y2/4 receptor induces superoxide production, which may be through activation of PKC or through the mobilization of intracellular calcium stores, which may modulate superoxide production.*

3.3.3 Conclusions

Exposure of microglia to the neurotransmitters glutamate, GABA or BzATP induces superoxide production through NADPH oxidase activation. Further investigations showed that inhibition of the group I mGluR, and activation of the group II and group III mGluRs induce superoxide production, whilst of the iGluRs, activation of the NMDA receptor, rather than the AMPA receptor mediates microglial superoxide production. Furthermore, activation of the $GABA_A$ receptor rather than the $GABA_B$ receptor, and activation of the P2Y2/4 receptor rather than the P2Y1 receptor promotes superoxide production in an NADPH oxidase dependent manner. There have been some indications from the literature as to possible mechanisms involved in NADPH oxidase activity and superoxide production as a result of modulation of neurotransmitter receptors, such as the involvement of TNFα, the activation of signalling pathways such as PKC and p38-MAPK, and also the involvement of multiple NADPH oxidase isoforms in superoxide and H_2O_2 production. These avenues of investigation have been explored in the next chapter, to determine which NADPH oxidase isoforms were modulated as a result of neurotransmitter receptor activation, and which signalling pathways were activated and contributed to NADPH oxidase activation, which may provide further insight into the role of microglial NADPH oxidase activation after modulation of neurotransmitter receptors.

Chapter 4

Neurotransmitter induction of NADPH oxidase isoforms and microglial signalling pathways implicated in NADPH oxidase isoform activity

4.1 Introduction and summary of results

The previous chapter showed that exposure of microglia to neurotransmitters or receptor modulators mediated NADPH oxidase activation. Microglial superoxide production induced as a consequence of NADPH oxidase activation can be protective or toxic (Hultqvist et al. 2009) depending on the NADPH oxidase isoform activated and the subsequent signalling pathways that are modulated (Chéret et al. 2008; Harrigan et al. 2008; Hultqvist et al. 2009). Microglial Nox1 activation is neurotoxic through the release of IL-1β (Chéret et al. 2008), and microglial Nox2 activation is associated with the progression of diseases such as AD (Block 2008) and PD (Gao et al. 2002), but also has important physiological functions in regulating intracellular processes such as the activity of transcription factors (Dröge 2002), and microglial proliferation (Mander et al. 2006). Furthermore, macrophage Nox2 activation and superoxide release down-regulates the T-cell response, which attenuates autoimmune disease progression (Hildeman et al. 2003; Hultqvist et al. 2009). Activation of the microglial Nox4 is associated with IL-6 production, however it is not known if this is protective or toxic, but is implicated in the regulation of the immune response (Li et al. 2009). Nox2 and Nox4 activation in the retina has been shown to be protective in models of cellular stress using serum starvation (Groeger et al. 2009), suggesting a dual role for these Nox isoforms. It was therefore considered important to investigate which NADPH oxidase isoforms were induced by neurotransmitter receptor modulation, to provide some insight as to whether activation of these isoforms may induce a protective or toxic microglial phenotype.

It was also important to investigate the signalling pathways implicated in NADPH oxidase isoform activation following modulation of microglial neurotransmitter receptors. Activation of microglial NADPH oxidase isoforms involves and induces the p44/42ERK (Miller et al. 2007) and p38MAPK (Sun et al. 2008) signalling cascades, which can promote a neurotoxic phenotype (Pocock & Liddle 2001). Activation of the MAPK signalling pathway is also

implicated in the progression of neurodegenerative diseases such as AD (Giovannini et al. 2002), ALS (Tortarolo et al. 2003), and PD (Miller et al. 2007). Furthermore, treatment of microglia with LPS mediates p38MAPK dependent NADPH oxidase activation and promotes phagocytosis (Sun et al. 2008), and activation of microglia with the HIV tat protein mediates p38MAPK dependent activation of the NADPH oxidase and neurotoxicity (Gupta et al. 2010; Song et al. 2011). Exposure of microglia to the herbicide paraquat, implicated in PD, also mediates p44/42ERK activation and NADPH oxidase activity (Miller et al. 2007). The MAPK signalling pathway is therefore a key regulator of microglial NADPH oxidase activation in neurodegenerative conditions.

This chapter therefore presents experiments detailing which NADPH oxidase isoforms are induced following modulation of neurotransmitter receptors, and also that p44/42ERK and p38MAPK signalling is involved in this superoxide production, which could provide information on the neurotoxic or neuroprotective roles of neurotransmitter induced ROS production in microglia.

The published findings that microglia express Nox1, Nox2 and Nox4 NADPH oxidase isoforms, and suggestions that the expression and activity of these isoforms can be modulated by the activity of different signalling pathways led to an investigation to determine which NADPH oxidase isoforms were induced following treatment of microglia with neurotransmitters and receptor agonists and antagonists. Glutamate was shown to induce Nox4 expression, and also enhanced Nox1 and Nox2 activity, whilst treatment with GABA or BzATP increased Nox1 and Nox4 expression and activity. The group I mGluR antagonist MTEP, the $GABA_A$ receptor agonist muscimol and the P2Y2/4 receptor agonist UTPγS enhanced Nox1 and Nox2 activity; whilst the mGluR3 agonist and the group III mGluR agonist increased Nox2 and Nox4 activity. NADPH oxidase isoform expression was only modulated by $GABA_A$ or P2Y2/4 receptor activation, suggesting that superoxide production

is controlled predominantly through kinase activation and subsequent phosphorylation of NADPH oxidase cytoplasmic domains following modulation of neurotransmitter receptors. Inhibition of the AMPA receptor induced Nox1 expression, whilst modulation of the NMDA receptor had little effect on NADPH oxidase isoform expression.

An investigation into the activity of the p38MAPK or p44/42ERK signalling pathway was performed to determine whether neurotransmitter receptor mediated ROS could affect microglial reactivity through cell signalling. Inhibition of p38MAPK attenuated NADPH oxidase induced superoxide production following $GABA_A$ receptor activation with muscimol, P2Y2/4 receptor activation with UTPγS, group III mGluR activation with L-AP4 or NMDA receptor activation; whilst inhibition of p44/42ERK inhibited NADPH oxidase activation and superoxide production following inhibition of the group I mGluR with MTEP or activation of mGluR3 with NAAG. Western blot analysis of phospho-p38MAPK or phospho-p44/42ERK showed that inhibition of superoxide production following modulation of the neurotransmitter receptors regulated the phosphorylation state of these signalling molecules, which would suggest a mechanism whereby MAPK signalling is dependent on NADPH oxidase activation, and that this signalling could also regulate NADPH oxidase induced superoxide production in microglia.

The data presented here therefore demonstrate that different NADPH oxidase isoforms are activated following modulation of different neurotransmitter receptors, and that MAPK signalling is modulated by neurotransmitter induced NADPH oxidase activation, which may have important consequences for microglial reactivity and neuronal survival.

4.2 Results

4.2.1 Neurotransmitters induce expression and activity of Nox1, Nox2 and Nox4

The previous chapter showed that neurotransmitters induced microglial superoxide production through NADPH oxidase activation; however these analyses did not provide information on which NADPH oxidase isoforms were expressed or activated. Different Nox isoforms preferentially produce either superoxide (Nox1 and Nox2) or H_2O_2 (Nox1 and Nox4). HPLC analysis of dHEth oxidation showed that treatment with glutamate or BzATP induced the production of the superoxide specific product 2-OH-E^+ suggestive of Nox2 activation, whilst GABA induced a significant increase in NADPH oxidase derived H_2O_2 production, suggestive of Nox4 activation. These findings lead to the investigation into neurotransmitter and receptor induced NADPH oxidase isoform expression and activity in microglia.

Nox1, Nox2 and Nox4 expression were investigated in primary microglia treated with the neurotransmitters glutamate (1 μM), GABA (100 μM) or BzATP (250 μM) using RT-PCR analysis (Fig. 4.1). Microglia were also treated with the positive control for microglial activation, LPS (1 μg/ml), the known NADPH oxidase activator PMA (10 ng/ml), or the NADPH oxidase inhibitor apocynin (10 μM). Microglia were treated for 24 h and RNA was extracted and reverse transcribed before PCR was performed using primers for Nox1, Nox2, Nox4, or β-actin which was used as a loading control (Fig. 4.1A). The RT-PCR was carried out three times, using three different microglial preparations, and densitometry was performed in which expression levels of each NADPH oxidase isoform were normalised to β-actin (Fig. 4.1B).

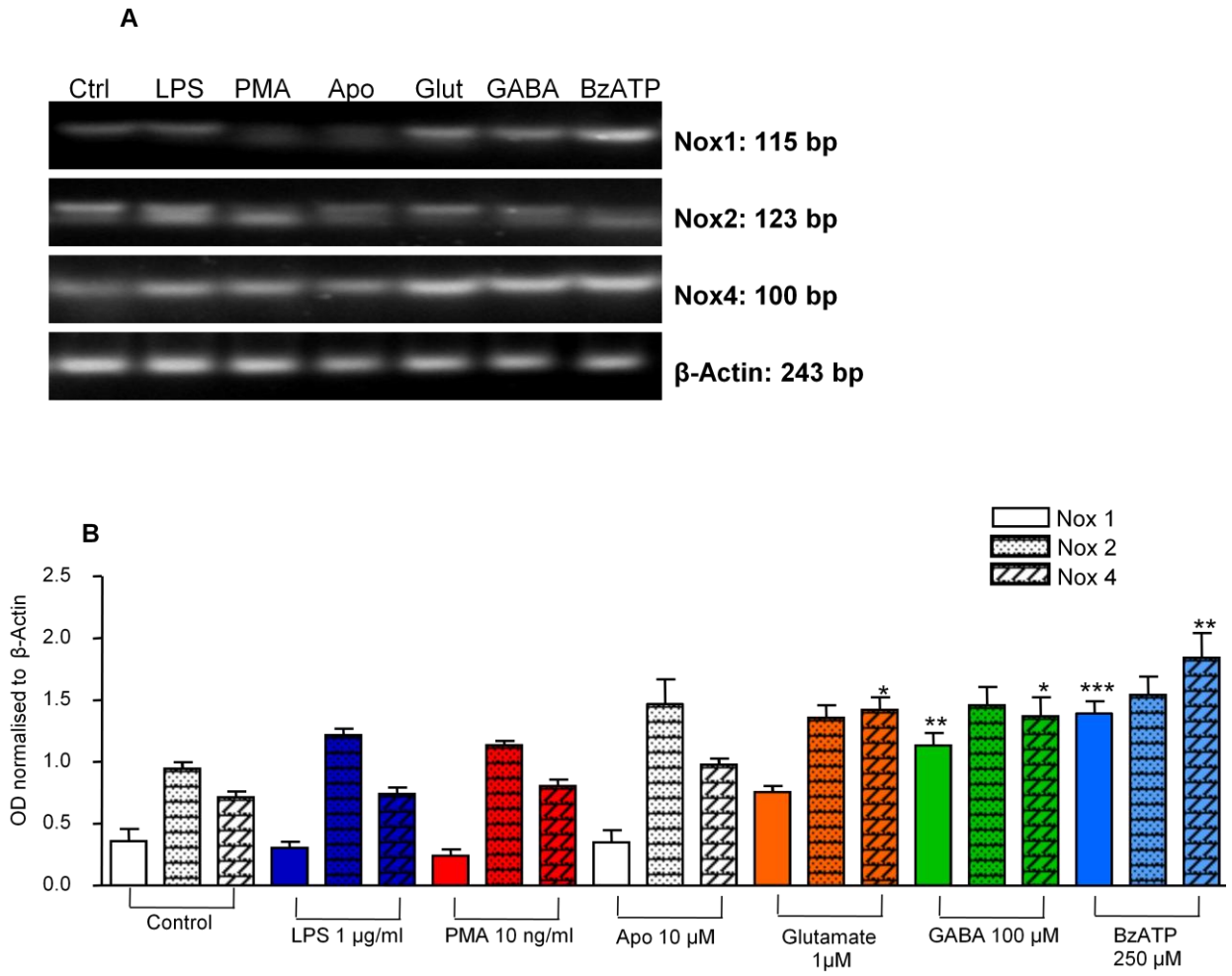

Figure 4.1 NADPH oxidase isoform expression in primary microglia treated with the neurotransmitters glutamate, GABA or BzATP. *Primary microglia were treated with Glutamate (1 µM), GABA (100 µM) or BzATP (250 µM), or the positive controls LPS (1 µg/ml) or PMA (10 ng/ml), or the NADPH oxidase inhibitor apocynin (10 µM) for 24 h. RNA was then extracted, and was reverse transcribed to cDNA. PCR was used to amplify Nox1, Nox2 or Nox4 transcribed genes, and β-actin was used as a loading control. PCR products were resolved on a 1% agarose gel by electrophoresis* ***(A)****. The experiment was carried out three times, and densitometry was performed* ***(B)****, in which expression of Nox1, Nox2 or Nox4 was normalised to β-Actin. Statistical analysis was performed on densitometry data, using a one way ANOVA, with post hoc analysis. All treatments were compared with control untreated cells, which is representative of basal NADPH oxidase isoform expression. *p<0.05, *p*<0.01, ***p<0.001. All data are n=3.*

Glutamate significantly increased microglial Nox4 expression, whilst treatment with GABA or BzATP significantly increased microglial Nox1 and Nox4 expression (Fig. 4.1B). These data therefore support the findings that Nox4 is predominantly regulated at the transcriptional level (Sorce & Krause 2009). As neurotransmitters have varying effects on Nox isoform expression, it was important to investigate the effects of glutamate, GABA or BzATP on the regulation of Nox isoform induced superoxide production and activity.

NADPH oxidase induced superoxide production is also regulated through the phosphorylation and translocation of cytoplasmic subunits, which is dependent on activation of signalling cascades. It was therefore important to investigate whether glutamate, GABA or BzATP could induce Nox1, Nox2 or Nox4 activity. Modulation of signalling pathways regulates NADPH oxidase activation and superoxide production, therefore NADPH oxidase isoform activation following neurotransmitter treatment was investigated by treating microglia with each of the neurotransmitters in the presence of different protein kinase inhibitors. To investigate the activation of Nox1/2, cells were treated with apocynin, as before, which prevents phosphorylation of the cytoplasmic p47phox subunit, which is part of the Nox1 and Nox2 NADPH oxidase, thereby inhibiting the assembly of the functional enzyme (Stefanska & Pawliczak 2008). To investigate Nox4 activation, cells were treated with neurotransmitters in the presence of thioridazine, which inhibits Nox4 enzymatic activity (Harrigan et al. 2008) through binding to the catalytic domain to inhibit electron transport across the Nox4 subunit (Serrander et al. 2007). To distinguish between Nox1 or Nox2 activation, microglia were treated with neurotransmitters in the presence of rottlerin, which is a PKCδ inhibitor shown to inhibit Nox1 expression and activation (Fan et al. 2005; Wei et al. 2010), or the PI3-K inhibitor wortmannin, which inhibits phosphorylation of the Nox2 specific p40phox (Kanai et al. 2001; Ellson et al. 2006). It was hoped that inhibition of

these kinases could show whether glutamate, GABA or BzATP induced signalling cascades that could modulate Nox1 or Nox2 activity.

BV2 and primary microglia were treated with glutamate (1 µM) (Fig. 4.2Ai, ii, iii), GABA (100 µM) (Fig. 4.2Bi, ii, iii) or BzATP (250 µM) (Fig. 4.2Ci, ii, iii) in the presence or absence of the inhibitors apocynin (10 µM), thioridazine (1 µM – Harrigan et al. 2008), rottlerin (10 µM – Fan et al. 2005) or wortmannin (1 µM – Kanai et al. 2001). The inhibitors did not induce microglial apoptosis or proliferation (Fig, 4.2Div) or modulate superoxide production or NADPH oxidase activity (Fig, 4.2Di, ii, iii) when applied to microglia alone. Superoxide production was measured by NBT analysis in BV2 microglia (Fig. 4.2Ai, Bi, Ci), or by dHEth fluorescence in primary microglia (Fig. 4.2Aii, Bii, Cii). NADPH oxidase activity was also assessed after treatment of BV2 microglia with neurotransmitters in the presence of the inhibitors (Fig. 4.2Aiii, Biii, Ciii).

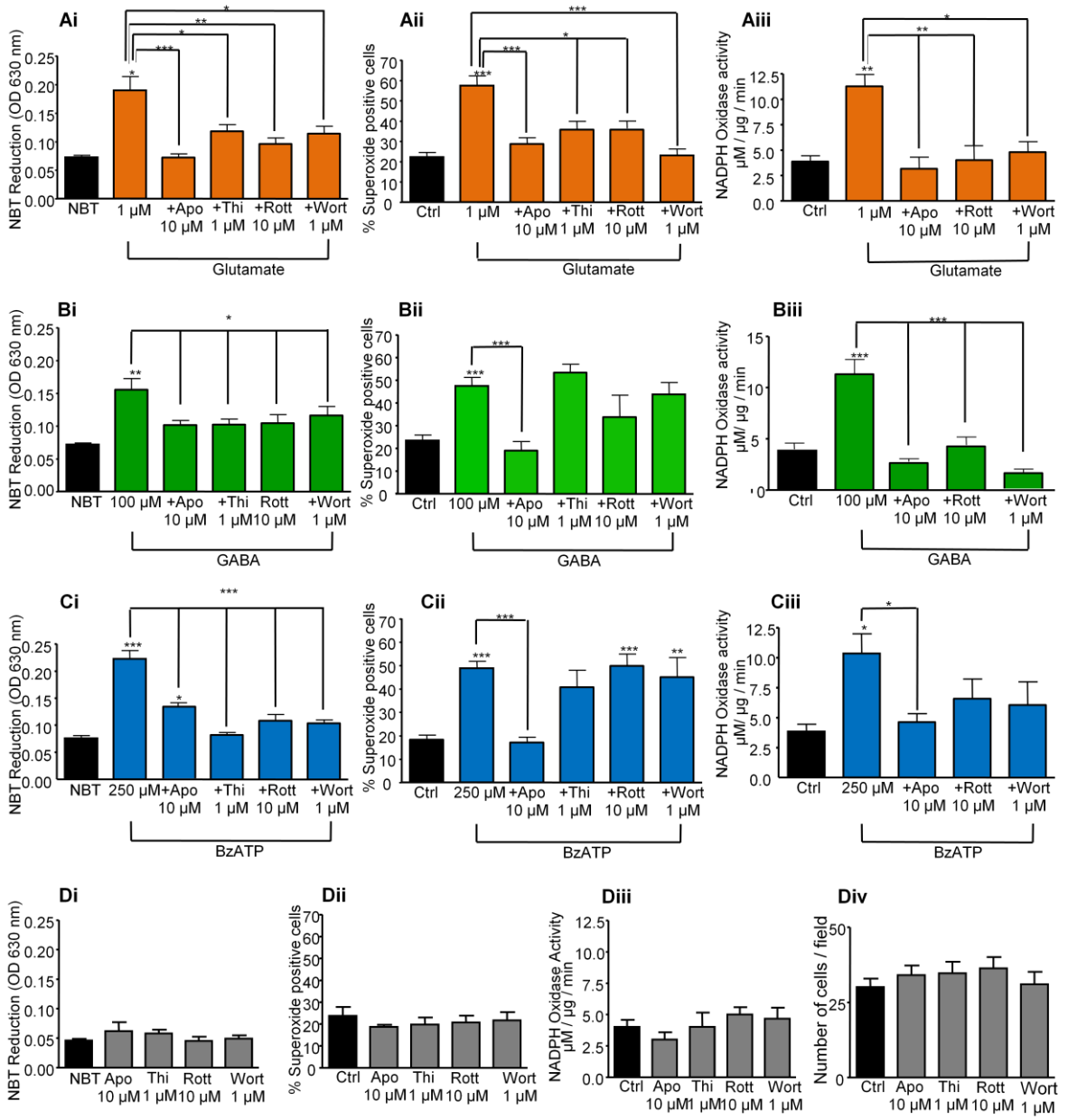

Figure 4.2 Analysis of NADPH oxidase isoform activity after treatment of BV2 or primary microglia with glutamate (Ai, ii, iii), GABA (Bi, ii, iii) or BzATP (Ci, ii, iii). *Superoxide production in BV2 microglia was analysed using the NBT assay* ***(Ai, Bi, Ci)****, and superoxide production in primary microglia was measured by dHEth fluorescence* ***(Aii, Bii, Cii).*** *NADPH oxidase activity was investigated in BV2 microglia using the activity assay* ***(Aiii, Ciii, Biii).*** *BV2 microglia were treated with glutamate (1 µM – panel A), GABA (100 µM – panel B) or BzATP (250 µM – panel C) in the presence or absence of apocynin (Apo, 10 µM), thioridazine (Thi, 1 µM), rottlerin (Rott, 10 µM) or wortmannin (Wort, 1 µM) for 4 h for the NBT assay* ***(Ai, Bi, Ci)*** *and the NADPH oxidase activity assay* ***(Aiii, Biii, Ciii)****.Primary microglia were treated as above for 24 h for the dHEth fluorescence assay* ***(Aii, Bii, Ciii)*** *in which microglia exhibiting red fluorescence in the nuclei were counted and expressed as a percent of superoxide positive microglia. NBT assays* ***(Di),*** *dHEth assays* ***(Dii)*** *and activity assays* ***(Diii)*** *were performed on BV2 and primary microglia treated with apocynin (10 µM), Thioridazine (1 µM), Rottlerin (10 µM) or Wortmannin (1 µM) alone to confirm that these inhibitors did not modulate superoxide production. To ensure that the inhibitors did not affect neuronal survival, cell counts were made of microglia treated with the inhibitors alone* ***(Div).*** *All data analysis was performed using a one way ANOVA with Tukey post-hoc analysis. Comparisons were made between cells treated with neurotransmitters alone and those co-treated with glutamate and inhibitors as indicated *p <0.05, *p*<0.01, ***p0.001. All data are n=3.*

Glutamate significantly increased NBT reduction in BV2 microglia in comparison to untreated controls (basal NBT reduction), which could be attenuated by co-treatment with apocynin, thioridazine, rottlerin or wortmannin (Fig. 4.2Ai). The most significant decrease in NBT reduction was seen upon co-treatment of BV2 microglia with glutamate and apocynin (***$p<0.001$), followed by co-treatment with rottlerin (**$p<0.01$), therefore suggesting that Nox1 and Nox2 are predominantly activated after glutamate treatment of BV2 microglia, and that exposure of BV2 microglia to glutamate induces PKCδ activity. Treatment of primary microglia with glutamate significantly elevated dHEth fluorescence, which was attenuated by co-treatment with apocynin, thioridazine, rottlerin or wortmamnnin (Fig. 4.2Aii). However, the most significant decrease in superoxide production was observed following treatment with glutamate and apocynin or wortmannin (***$p<0.001$), suggesting the involvement of Nox2 in glutamate induced superoxide production in primary microglia, and indicating that glutamate induces PI3-K activation in primary microglia. In agreement with the NBT assay, treatment of BV2 microglia with glutamate and apocynin or rottlerin induced the most significant decrease in NADPH oxidase activity (**$p<0.01$), however co-treatment of BV2 microglia with glutamate and wortmannin also significantly reduced enzymatic activity (*$p<0.05$) (Fig. 4.2Aiii). Together, these data show that glutamate induces superoxide production through Nox1 and Nox2 activation.

GABA induced NBT reduction was attenuated by co-treatement with GABA and apocynin, thiorodazine, rottlerin or wortmannin (Fig. 4.2Bi), implicating Nox1, 2, and 4 in GABA induced ROS production. Treatment of primary microglia with GABA significantly increased the percentage of dHEth fluorescent microglia, which was attenuated by co-treatment of primary microglia with apocynin (Fig. 4.2Bii), suggesting that GABA preferentially activates Nox2. This was supported by the activity assay (Fig. 4.2Biii), which showed a significant decrease in NADPH oxidase activity following co-treatment of BV2 microglia with GABA

and apocynin, rottlerin or wortmannin, implicating Nox1 and Nox2 in GABA induced ROS production. These data suggest that in BV2 microglia, GABA induces PI3-K and PKCδ activation to induce Nox1 and Nox2 mediated superoxide production, as well as inducing Nox4 activity, whereas in primary microglia, GABA mediates Nox1 and Nox2 activation in a PKCδ and PI3-K independent manner.

Treatment of BV2 microglia with BzATP significantly increased NBT reduction which was attenuated by co-treatment of BV2 microglia with apocynin, thioridazine, rottlerin or wortmannin, suggesting that all three NADPH oxidase isoforms are implicated in BzATP induced superoxide production in BV2 microglia (Fig. 4.2Ci). BzATP induced dHEth fluorescence could only be attenuated by co-treatment with apocynin (Fig. 4.2Cii), suggesting that BzATP only induces Nox2 activation in primary microglia, and that this is independent of PI3-K or PKC signalling. Co-treatment of primary microglia with BzATP and rottlerin or wortmannin significantly increased superoxide production when compared with control untreated microglia (Fig. 4.2Cii). Inhibition of NADPH oxidase isoforms following BzATP treatment may therefore promote further superoxide production through increased activation of other NADPH oxidase isoforms or other ROS producing enzymes. The NADPH oxidase activity assay (Fig. 4.2Ciii) agreed with the imaging data, showing a significant decrease in NADPH oxidase activity upon co-treatment of BV2 microglia with BzATP and apocynin, suggesting that Nox2 is predominantly activated by BzATP.

In summary, glutamate induced microglial Nox4 expression and Nox1 and Nox2 activation in a PKCδ and PI3-K dependent manner; GABA increased Nox1 and Nox4 expression and activity; and BzATP up-regulated Nox1 and Nox4 expression, and Nox1/2 activity. GABA or BzATP induced Nox1 and Nox2 activation in primary microglia was independent of PI3-K or PKCδ activation. These data lead on to investigations to determine which NADPH oxidase isoforms could be activated by modulation of neurotransmitter receptors.

4.2.2 Neurotransmitter receptor modulation affects the expression and activity of Nox1, Nox2, and Nox4

Initially, RT-PCR was used to examine Nox1, Nox2 and Nox4 expression in primary microglia treated with the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 μM), the mGluR group III agonist L-AP4 (100 μM), the $GABA_A$ receptor agonist muscimol (50 μM) or the P2Y2/4 receptor agonist UTPγS (100 μM) in the presence or absence of apocynin (10 μM) for 24 h (Fig. 4.3). PCR was used to amplify Nox1, Nox2, Nox4 and β-actin as a loading control (Fig. 4.3A). The RT-PCR was performed three times, using three separate microglial preparations, and densitometry was performed, in which expression levels of Nox1 (Fig. 4.3Bi), Nox2 (Fig. 4.3Bii) or Nox4 (Fig. 4.3Biii) were normalised to the β-actin loading control.

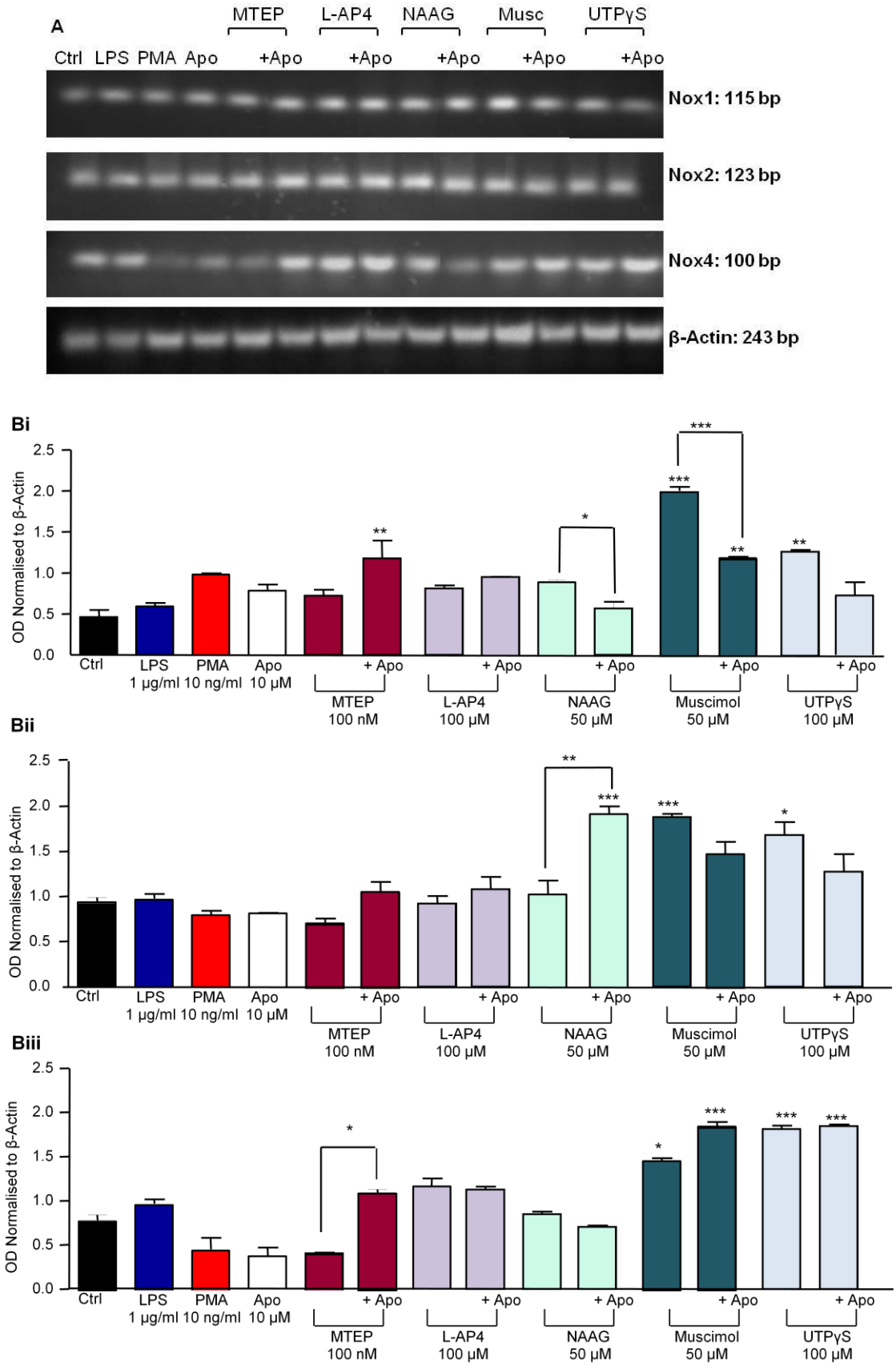

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Figure 4.3 Nox1, Nox2 and Nox4 expression in primary microglia after treatment with neurotransmitter receptor agonists and antagonists. *Primary microglia were treated with the controls LPS (1 µg/ml), PMA (10 ng/ml) or apocnin (10 µM), or the mGluR group I receptor antagonist MTEP (100 nM), the group III agonist L-AP4 (100 µM), the mGluR3 agonist NAAG (50 µM), the $GABA_A$ receptor agonist muscimol (50 µM) or the P2Y2/4 receptor agonist UTPγS (100 µM) in the presence or absence of apocynin (10 µM) for 24 h. RNA was then extracted and reverse transcribed to cDNA. PCR was performed using primers for Nox1, Nox2 or Nox4, as well as primers for the loading control β-actin. Products were subjected to electrophopresis on a 1% agarose gel, with EthBr for visualisation* ***(A).*** *The experiment was conducted in triplicate using three separate microglial preparations, to enable densitometry to be performed. Expression of Nox1* ***(Bi)****, Nox2* ***(Bii)*** *and Nox4* ***(Biii)*** *was normalised to β-actin, to quantify expression of each NADPH oxidase isoform. Densitometry was analysed by one way ANOVA with Tukey post-hoc analysis, comparing all treatments with control un-treated cells, and comparisons were also made between cells treated with neurotransmitter receptor agonists / antagonists alone or in the presence of apocynin as indicated. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

Muscimol or UTPγS treatment significantly elevated microglial Nox1 expression (Fig. 4.3Bi), and in the case of muscimol, Nox1 expression could be attenuated by co-treatment of microglia with muscimol and apocynin, suggesting the presence of a feedback mechanism in which attenuation of superoxide production inhibits Nox gene expression. Treatment of microglia with the mGluR3 agonist NAAG did not significantly elevate Nox1 expression, however, co-treatment of microglia with NAAG and apocynin significantly decreased Nox1 expression when compared with Nox1 expression elicited by NAAG treatment alone (Fig. 4.3Bi).

Nox2 expression was significantly up-regulated by treatment of microglia with muscimol or UTPγS (Fig. 4.3Bii). NAAG plus apocynin treatment of microglia also significantly elevated Nox2 expression when compared with control untreated cells or NAAG treatment alone. This could suggest that a decrease in superoxide production through inhibition of enzymatic activity may promote Nox2 expression to regulate superoxide levels in microglia.

Treatment of primary microglia with muscimol or UTPγS significantly elevated Nox4 expression, however, this was not modulated by co-treatment with apocynin (Fig. 4.3Biii). Treatment of microglia with MTEP and apocynin significantly elevated Nox4 expression when compared with control untreated cells, and also when compared with MTEP treatment alone (Fig. 4.3Biii), again suggesting the presence of a regulatory mechanism in which inhibition of receptor induced Nox1 or Nox2 expression with apocynin may enhance Nox4 expression.

As modulation of microglial neurotransmitter receptors had little effect on NADPH oxidase isoform expression, it was important to determine whether neurotransmitter receptor modulation could affect NADPH oxidase isoform activity. BV2 and primary microglia were treated with the receptor agonists or antagonists in the presence or absence of the Nox1/2

inhibitor apocynin (10 μM), the Nox4 inhibitor thioridazine (1 μM), the PKCδ inhibitor rottlerin (10 μM), or the PI3-K inhibitor wortmannin (1 μM) before superoxide production and NADPH oxidase activity was measured.

To asses whether modulation of mGluRs mediated the activity of different NADPH oxidase isoforms, BV2 microglia were treated with MTEP (100 nM) (Fig. 4.4Ai, ii, iii), NAAG (50 μM) (Fig. 4.4Bi, ii, iii) or L-AP4 (100 μM) (Fig. 4.4Ci, ii, iii) in the presence or absence of apocynin (10 μM), thioridazine (1 μM), rottlerin (10 μM), or wortmannin (1 μM). The NBT assay was used to investigate superoxide production after inhibition of NADPH oxidase isoforms (Fig. 4.4Ai, Bi, Ci). Superoxide production in primary microglia was also investigated using dHEth fluorescence microscopy, following treatment of microglia with MTEP (Fig. 4.4Aii), NAAG (Fig. 4.4Bii) or L-AP4 (Fig. 4.4Cii) in the presence or absence of the inhibitors apocynin, thioridazine, rottlerin or wortmannin. NADPH oxidase activity was also investigated in BV2 microglia after 4 h incubation with the aforementioned conditions (Fig. 4.4Aiii, Biii, Ciii).

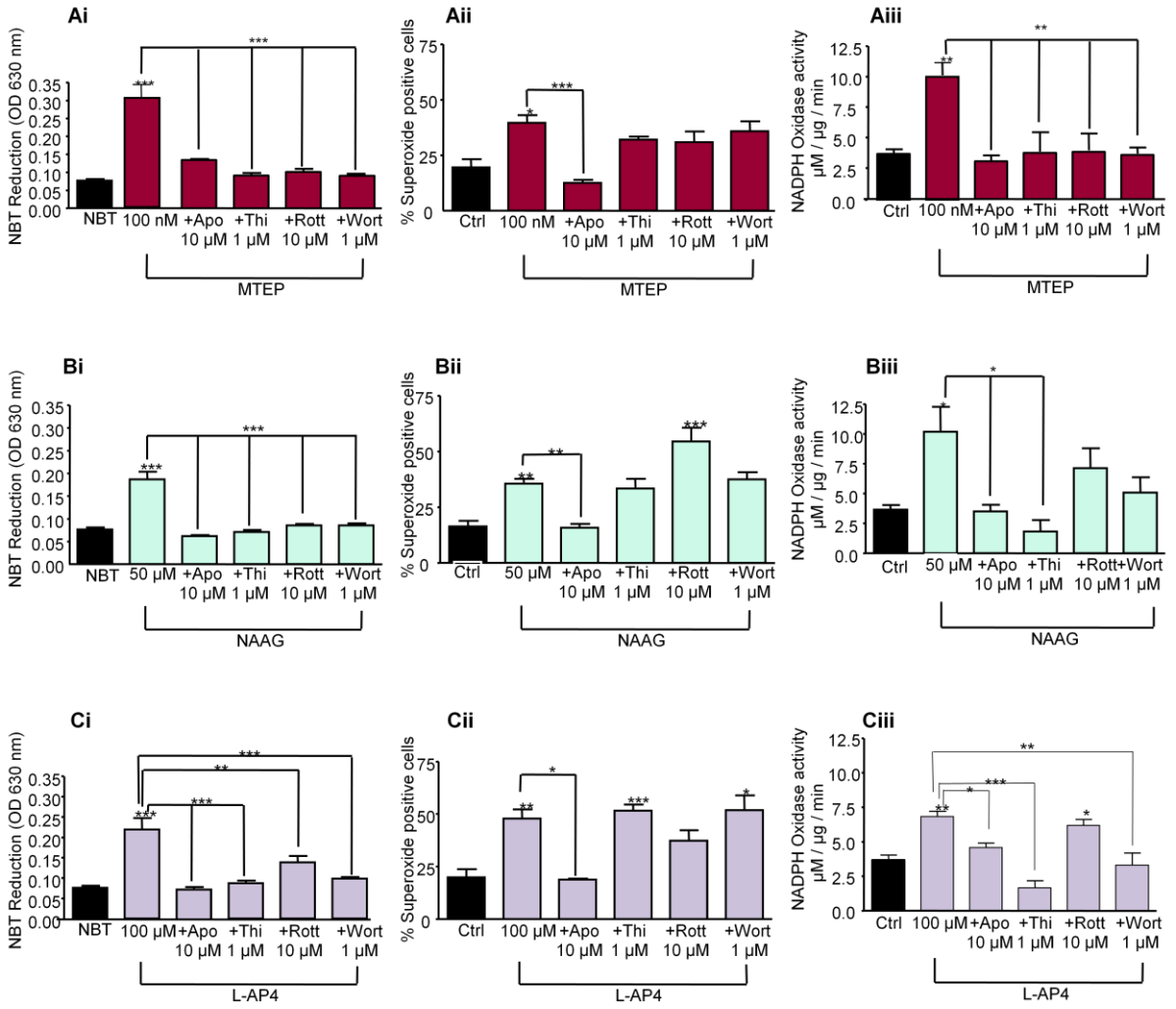

Figure 4.4 Modulation of NADPH oxidase isoform activity after treatment of microglia with the group I mGluR antagonist MTEP (Ai, ii, iii), the mGluR3 agonist NAAG (Bi, ii, iii) or the group III mGluR agonist L-AP4 (Ci, ii, iii). *BV2 microglia were treated with the group I mGluR agonist MTEP (100 nM)* ***(Ai, ii, iii),*** *the mGluR3 agonist NAAG* ***(Bi, ii, iii,),*** *or the group III mGluR agonist L-AP4* ***(Ci, ii, iii)*** *in the presence or absence of the NADPH oxidase inhibitors apocynin (apo, 10 µM), thioridazine (Thi, 1 µM), rottlerin (Rott, 10 µM) or wortmannin (Wort, 1 µM) for 4 h before NBT reduction was measured* ***(Ai, Bi, Ci).*** *Dihydroethidium fluorescence was used to investigate superoxide production in primary microglia treated as above for 24 h, and the percentage superoxide positive cells were determined* ***(Aii, Bii, Cii)*** *from images taken. NADPH oxidase activity was also analysed after treatment of BV2 microglia as described for 4 h* ***(Aiii, Biii, Ciii)****. For all analyses, data were analysed using a one way ANOVA with Tukey post-hoc analysis, in which comparisons were made between treated and control un-treated cells, and also between treatments in the presence or absence of inhibitors as indicated. *p<0.05, *p*<0.01, ***p<0.001. All data are n=3.*

Antagonism of the group I mGluR with MTEP significantly increased NBT reduction (Fig. 4.4Ai) and dHEth fluorescence (Fig. 4.4Aii) when compared with control untreated microglia. The increased NBT reduction after treatment of BV2 microglia with MTEP was inhibited by co-treatment with apocynin, thioridazine, rottlerin or wortmannin (Fig. 4.4Ai); suggesting that MTEP induces the activity of all NADPH oxidase isoforms in BV2 microglia through PKCδ and PI3-K activation. However, the MTEP induced increase in dHEth fluorescence in primary microglia was only attenuated by co-treatment with apocynin (Fig. 4.4Aii), suggesting that only Nox1/2 is activated by inhibition of the group I mGluR, which is independent of PKCδ or PI3-K activation. In contrast, MTEP induced NADPH oxidase activity was significantly attenuated by all inhibitors tested, lending support to the NBT analysis (Fig. 4.4Aiii). In BV2 microglia, inhibition of the group I mGluRs induces superoxide production through activation of PKCδ and PI3-K to induce Nox1 and Nox2 activation, as well as inducing Nox4 activity, whereas in primary microglia, MTEP induced superoxide production is a result of Nox1/2 activation only, and is independent of PKC and PI3-K signalling.

The mGluR3 mediated NBT reduction was significantly attenuated by co-treatment of cells with apocynin, thioridazine, rottlerin or wortmannin (Fig. 4.4Bi), therefore suggesting that in BV2 microglia NAAG induces superoxide production through Nox1 and Nox2 activation in a PKCδ and PI3-K dependent manner, and also promotes Nox4 activation. NAAG induced dHEth fluorescence in primary microglia could only be significantly reduced by co-treatment with apocynin, suggesting that superoxide production is a consequence of Nox1 and Nox2 activation only, and is independent of PI3-K and PKCδ activation (Fig. 4.4Bii). Treatment of primary microglia with NAAG and the PKCδ inhibitor rottlerin significantly increased dHEth fluorescence when compared with control untreated cells (Fig.4.4Bii) which suggests that Nox1 inhibition enhances the activity of other NADPH oxidase isoforms. The activity

analysis correlated with the primary microglial dHEth fluorescence analysis, showing that treatment of BV2 microglia with NAAG significantly increased NADPH oxidase activity, which could be attenuated by co-treatment with apocynin or thioridazine (Fig. 4.4Biii). These data therefore suggest that NAAG induced superoxide production is mediated by Nox1 and Nox4 activation.

L-AP4 treatment significantly elevated NBT reduction, which was attenuated by co-treatment of BV2 microglia with each inhibitor tested (Fig. 4.4Ci). Co-treatment of BV2 microglia with L-AP4 and wortmannin, thioridazine or apocynin induced the most significant decrease in NBT reduction (***$p<0.001$), suggesting that activation of the group III mGluRs in BV2 microglia induces Nox2 and Nox4 activation through PI3-K activation. These findings correlated with NADPH oxidase activity in BV2 microglia (Fig. 4.4Ciii). L-AP4 induced superoxide production in primary microglia could only be attenuated by co-treatment of cells with apocynin (Fig. 4.4Cii), and co-treatment of microglia with L-AP4 and thioridazine or wortmannin significantly elevated the number of superoxide positive microglia (Fig. 4.4Cii). This could suggest that in primary microglia, inhibition of one isoform following activation of the group III mGluR may induce superoxide production through the enhanced activation of other NADPH oxidase isoforms.

It was next important to investigate whether modulation of the $GABA_A$ or P2Y2/4 receptors induced Nox1, Nox2 or Nox4 mediated superoxide production. BV2 and primary microglia were treated with muscimol (50 μM) (Fig. 4.5Ai, ii, iii) or UTPγS (100 μM) (Fig. 4.5Bi, ii, iii) in the presence or absence of apocynin (10 μM), thioridazine (1 μM), rottlerin (10 μM) or wortmannin (1 μM). Cells were assessed for superoxide production using the NBT assay (Fig. 4.5Ai, Bi), and superoxide production was analysed in primary microglia using dHEth fluorescence microscopy (Fig. 4.5Aii, Bii). NADPH oxidase activity in BV2 microglia was assessed using the activity assay after treatment as described for 4 h (Fig. 4.5Aiii, Biii).

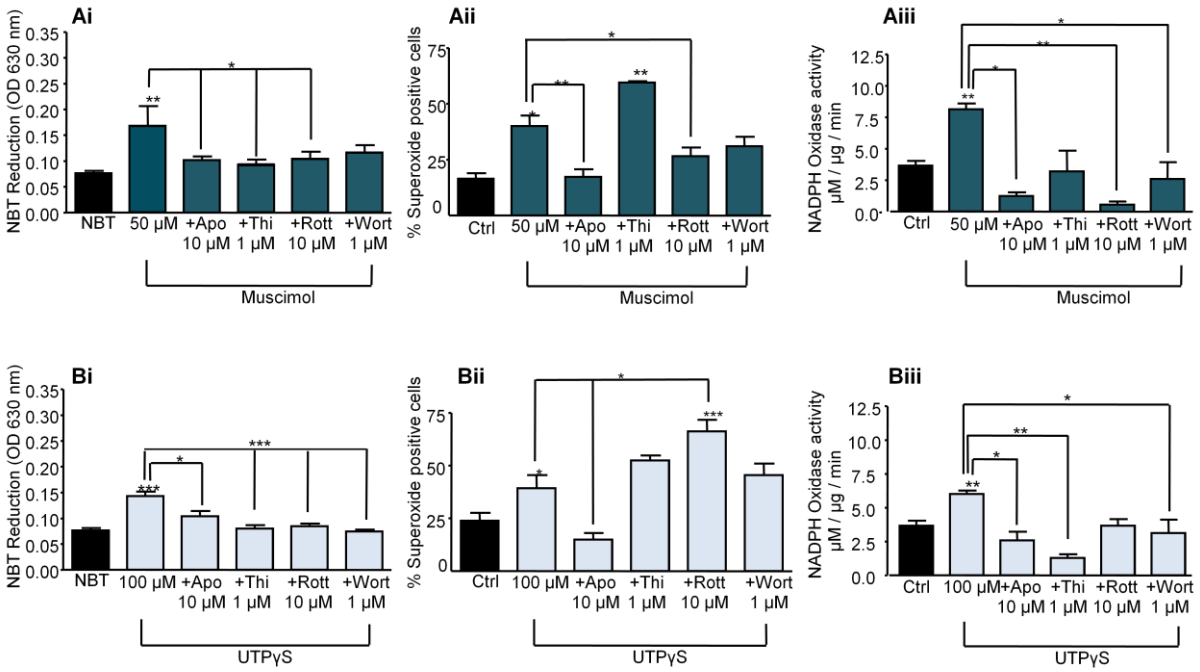

Figure 4.5 Modulation of NADPH oxidase isoform activity after treatment of microglia with the $GABA_A$ receptor agonist muscimol (Ai, ii, iii) or the P2Y2/4 receptor agonist UTPγS (Bi, ii,iii). *BV2 microglia were treated with the $GABA_A$ receptor agonist muscimol (50 µM)* ***(Ai, ii, iii)*** *or the P2Y2/4 receptor agonist UTPγS* ***(Bi, ii, iii)*** *in the presence or absence of the NADPH oxidase inhibitors apocynin (apo, 10 µM), Thioridazine (Thi, 1 µM), rottlerin (Rott, 10 µM) or wortmannin (Wort, 1 µM) for 4 h before NBT reduction was measured* ***(Ai, Bi).*** *Dihydroethidium fluorescence was used to investigate superoxide production in primary microglia treated as above for 24 h, and the percent superoxide positive cells were determined* ***(Aii Bii)*** *from images taken.* ***NADPH oxidase activity*** *was also analysed after treatment of BV2 microglia as described for 4 h* ***(Aiii, Biii)****. For all analyses, data were analysed using a one way ANOVA with Tukey post-hoc analysis, in which comparisons were made between treated and control, untreated cells, and also between treatments in the presence or absence of inhibitors as indicated. *p<0.05, **p<0.01, ***p<0.001.All data are n=3.*

Muscimol significantly increased NBT reduction when compared with untreated controls, which was attenuated by co-treatment with apocynin, thioridazine or rottlerin in BV2 microglia (Fig. 4.5Ai). Muscimol induced superoxide production in primary microglia was attenuated by co-treatment with apocynin or rottlerin (Fig. 4.5Aii), in agreement with the NADPH oxidase activity assay, demonstrating that muscimol induced NADPH oxidase activity could be reduced most significantly by co-treatment with rottlerin (**$p<0.01$) (Fig. 4.5Aiii). Furthermore, apocynin or wortmannin also attenuated muscimol induced NADPH oxidase activity (Fig. 4.5Aiii). These data therefore suggest that $GABA_A$ receptor activation induces NADPH oxidase derived superoxide production through the activation and expression of Nox1 and Nox2, in a PKCδ dependent manner.

UTPγS induced NBT reduction was significantly attenuated by apocynin, thioridazine, rottlerin or wortmannin (Fig. 4.5Bi). In contrast, UTPγS induced dHEth fluorescence in primary microglia could only be attenuated by co-treatment of cells with apocynin (Fig. 4.5Bii) and this treatment also induced the most significant attenuation of NADPH oxidase activity (**$p<0.01$) (Fig. 4.5Biii). Co-treatment of primary microglia with UTPγS and rottlerin significantly increased dHEth fluorescence when compared with control untreated cells and those treated with UTPγS alone (Fig. 4.5Bii), and co-treatment of BV2 microglia with UTPγS and rottlerin had no effect on NADPH oxidase activity (Fig. 4.5Biii). Treatment of BV2 microglia with UTPγS and wortmannin significantly reduced NADPH oxidase activity (Fig. 4.5Biii). These data together suggest that modulation of the P2Y2/4 receptor induces superoxide production Nox1 and Nox2 activation, and in BV2 microglia this is dependent on PI3-K activation.

The expression and functional analysis therefore showed that antagonism of the group I mGluR with MTEP, activation of the $GABA_A$ receptor with muscimol, and activation of the P2Y2/4 receptor with UTPγS induced superoxide production through Nox1 and Nox2

activation, whereas activation of the group II mGluR3 with NAAG and activation of the group III mGluR with L-AP4 induced Nox2 and Nox4 derived superoxide production.

Nox1, Nox2 and Nox4 expression were next investigated after modulation of the microglial iGluRs, as it was shown in the previous chapter that activation of the microglial NMDA receptor elevated superoxide production, whilst modulation of the AMPA receptor had no effect on ROS production. Primary microglia were treated with NMDA (100 μM) or MK-801 (10 μM) either alone, in combination, or in the presence of apocynin (10 μM); or were treated with the AMPA receptor agonist QA (10 μM) or the antagonist CNQX (10 μM) either alone, in combination, or in the presence of apocynin (10 μM). PCR was performed using primers for Nox1, Nox2 and Nox4, as well as β-actin as a loading control (Fig. 4.6A). The PCR was conducted in triplicate, using three separate microglial preparations, and densitometry was performed, in which Nox1 (Fig. 4.6Bi), Nox2 (Fig. 4.6Bii) and Nox4 (Fig. 4.6Biii) expression was normalised to β-actin.

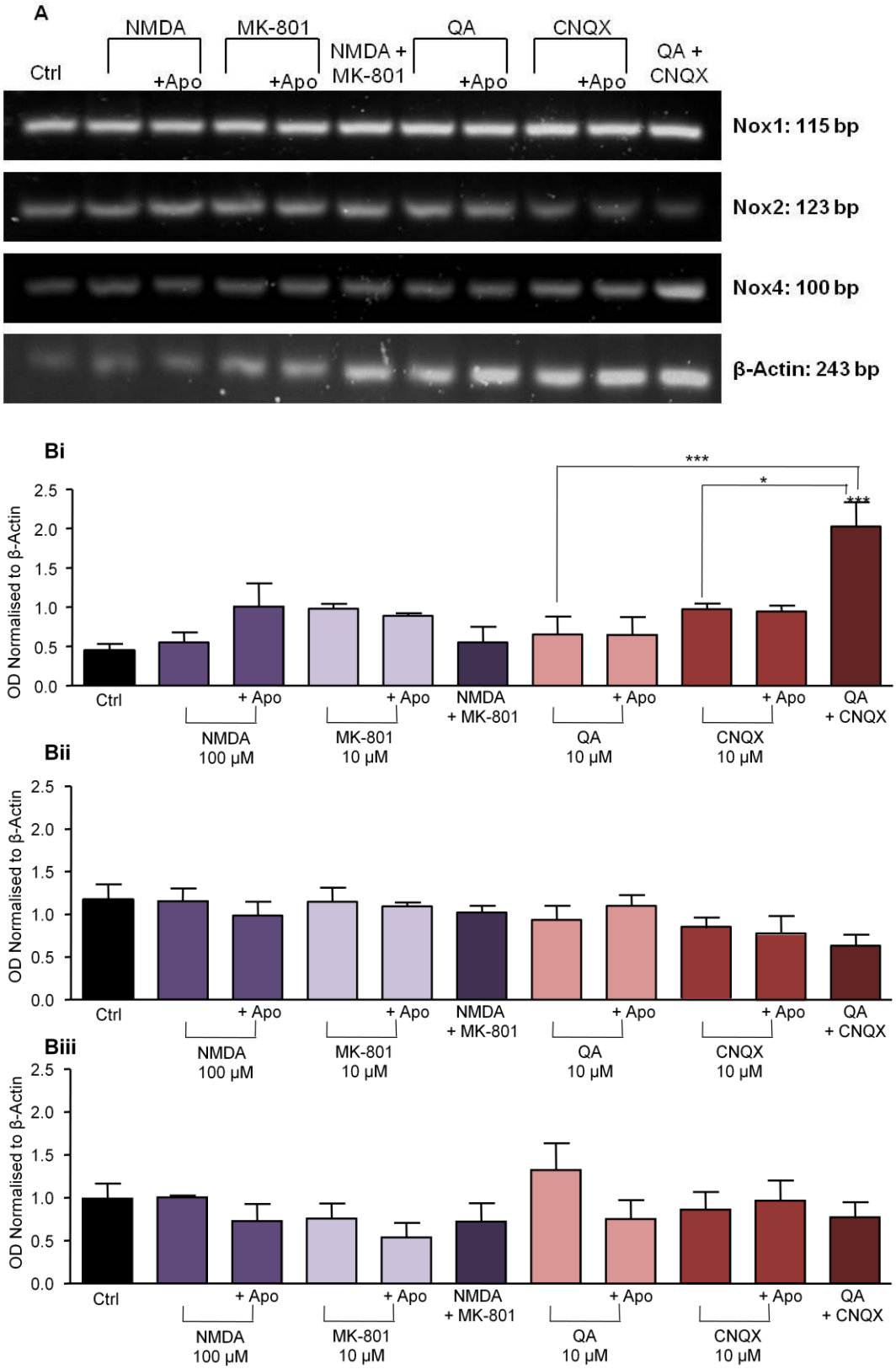

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Figure 4.6 Nox1, Nox2 and Nox4 expression in primary microglia after treatment with iGluR receptor agonists and antagonists. *Primary microglia were treated with the NMDA receptor agonist NMDA (100 µM), or the antagonist MK-801 (10 µM) either alone, in combination or in the presence of apocynin (10 µM) for 24 h. Microglia were also treated with the AMPA receptor agonist QA (10 µM), or the antagonist CNQX (10 µM) either alone, in combination or in the presence of apocynin for 24 h. RNA was then extracted and reverse transcribed to cDNA. PCR was performed using primers for Nox1, Nox2 or Nox4, as well as the loading control β-actin. Products were subjected to electrophopresis on a 1% agarose gel, with EthBr for visualisation* ***(A).*** *The experiment was conducted in triplicate using three separate microglial preparations, to enable densitometry to be performed. Expression of Nox1* ***(Bi)****, Nox2* ***(Bii)*** *and Nox4* ***(Biii)*** *was normalised to β-actin, to quantify expression of each NADPH oxidase isoform. Densitometry was analysed by one way ANOVA with Tukey post-hoc analysis, comparing all treatments with control un-treated cells, and making comparisons between cells treated with neurotransmitter receptor agonists / antagonists alone or in the presence of apocynin as indicated. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

Modulation of the NMDA receptor did not significantly alter Nox1 (Fig. 4.6Bi), Nox2 (Fig. 4.6Bii) or Nox4 (Fig. 4.6Biii) expression when compared with control untreated cells. Co-treatment of microglia with the AMPA receptor agonist QA and the antagonist CNQX significantly increased Nox1 expression (Fig. 4.6Biii) when compared with control untreated microglia, and also when compared with CNQX or QA treatments alone. Activation of the NMDA receptor was previously shown to elevate superoxide production, however as NMDA did not increase NADPH oxidase isoform expression, it could be suggested that activation of the NMDA receptor may induce phosphorylation of cytoplasmic subunits through the activation of signalling pathways to modulate NADPH oxidase activity rather than expression.

4.2.3 MAPK signalling in neurotransmitter receptor modulated NADPH oxidase activation

Activation of the microglial MAPK signalling cascade is implicated in several neurodegenerative conditions, and MAPK signalling can be regulated by the redox state of microglia, which is perturbed following microglial activation (Pawate et al. 2004). In microglia, MAPK signalling is modulated by exposure to growth factors, modulation of GPCR's, cellular stresses or inflammatory mediators which promote the phosphorylation and activation of p38MAPK or p44/42ERK. Activation of microglial p38MAPK with Aβ induces an inflammatory phenotype, characterised by the release of cytokines, which can feedback onto microglia perpetuating inflammation and enhancing disease progression (Pocock & Liddle 2001). Activation of the microglial p44/42ERK is detrimental to neuronal survival, and treatment with LPS, Aβ or inflammatory cytokines can induce a neurotoxic phenotype through up-regulation of ERK1/2 (Koistinaho & Koistinaho 2002). Furthermore, modulation of the microglial cannabinoid receptor type 2 (CBR2) can down-regulate ERK activation following exposure to LPS, and is protective against neurotoxicity (Romero-Sandoval et al.

2009). In addition, anti-inflammatory drugs attenuate microglial Aβ induced p44/42ERK phosphorylation, which prevents the cognitive deficits associated with AD in mouse models (Zhu et al. 2008).

As the p44/42ERK and p38MAPK signalling molecules are implicated in the activation of the microglial NADPH oxidase (Miller et al. 2007; Sun et al. 2008) and microglial induced neurotoxicity, it was considered important to investigate whether modulation of neurotransmitter receptors could mediate NADPH oxidase activation through the MAPK signalling pathway, which may provide important information on the role of receptor induced ROS on microglial reactivity.

To investigate the involvement of p44/42ERK and p38MAPK activity in neurotransmitter receptor induced NADPH oxidase activation and ROS production, the NBT assay, dHEth fluorescence imaging and the NADPH oxidase activity assay were used following treatment of microglia with the receptor modulators and MAPK inhibitors. BV2 and primary microglia were treated with the mGluR group I receptor antagonist MTEP (100 nM) (Fig. 4.7Ai, ii, iii), the mGluR3 agonist NAAG (50 μM) (Fig. 4.7Bi, ii, iii) or the mGluR group III agonist L-AP4 (100 μM) (Fig. 4.7Ci, ii, iii), either alone, or in combination with apocynin (10 μM), the p44/42ERK inhibitor PD-98059 (PD, 30 μM (Kim et al. 2002)) or the p38MAPK inhibitor SB-203580 (SB, 10 μM (Han et al. 2002)), which were shown not to modulate superoxide production (Fig. 4.7Di, ii, iii) or microglial survival (Fig. 4.7Div). Superoxide production was assessed by NBT reduction in BV2 (Fig. 4.7Ai, Bi, Ci), and dHEth fluorescence microscopy in primary (Fig.4.7Aii, Bii, Cii) microglia. NADPH oxidase activity was assessed in BV2 microglia using the NADPH oxidase activity assay (Fig. 4.7Aiii, Biii, Ciii).

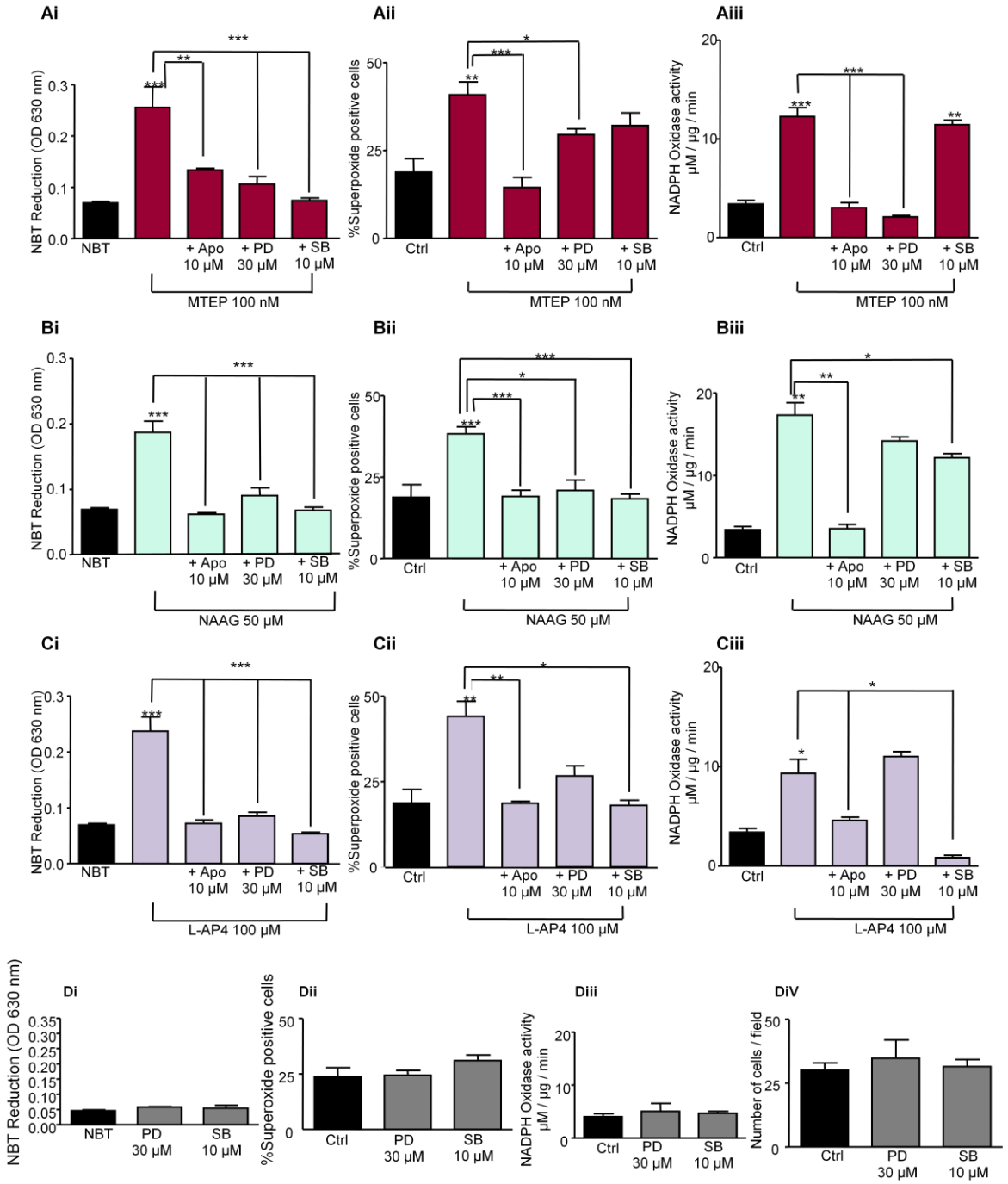

Figure 4.7 Inhibition of p44/42ERK or p38MAPK attenuates superoxide production and NADPH oxidase activity following modulation of microglial mGluRs. *Primary and BV2 microglia were treated with the mGluR group I antagonist MTEP (100 nM – panel A), the mGluR3 agonist NAAG (50 µM – panel B) or the mGluR group III agonist L-AP4 (100 µM – panel C) in the presence or absence of apocynin (10 µM), the p44/42ERK inhibitor PD-90859 (PD – 30 µM), or the p38MAPK inhibitor SB-203580 (SB – 10 µM). Superoxide production was analysed in BV2 microglia by NBT reduction* ***(Ai, Bi, Ci)****, and in primary microglia using dHEth fluorescence and imaging of superoxide positive cells* ***(Aii, Bii, Cii,),*** *whilst NADPH oxidase activity was analysed in BV2 microglia using the NADPH oxidase activity assay* ***(Aiii, Biii, Ciii).*** *Superoxide production and NADPH oxidase activity was assessed following treatment of microglia with PD-98059 or SB-203580 alone* ***(Di, Dii, Diii,)*** *and microglial proliferation and survival was measured by cell counts* ***(Div).*** *All data were analysed by one way ANOVA with Tukey post-hoc analysis, and comparisons were made between treatments and un-treated control cells, and also between treatments alone and co-treatment with the inhibitors as indicated. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

Attenuation of p38MAPK and p44/42ERK significantly reduced mGluR group I mediated NBT reduction in BV2 microglia (***$p<0.001$) (Fig. 4.7Ai). In primary microglia however, only inhibition of p44/42ERK could attenuate MTEP induced superoxide production (Fig. 4.7Aii), which correlated with the NADPH oxidase activity assay (Fig. 4.7Aiii). Co-treatment of BV2 microglia with MTEP and the p38MAPK inhibitor significantly increased NADPH oxidase activity thereby suggesting that MTEP induced NADPH oxidase activity and subsequent superoxide production is mediated through p44/42ERK activation.

Activation of the BV2 and primary microglial mGluR3 with NAAG elevated NBT reduction and dHEth fluorescence respectively, which was attenuated by co-treatment with the p44/42ERK inhibitor or the p38MAPK inhibitor (Fig. 4.7Bi, Bii). In primary microglia however, p38MAPK inhibition decreased NAAG induced superoxide production more significantly (***$p<0.001$) than inhibition of p44/42ERK (*$p<0.05$). This correlated with the NADPH oxidase activity analysis, which showed that NAAG induced NADPH oxidase activity was attenuated by p38MAPK inhibition, whilst p44/42ERK inhibition had no effect on NAAG induced NADPH oxidase activity (Fig. 4.7Biii). These data show that mGluR3 induced superoxide production is mediated by p38MAPK activation.

NBT reduction induced by activation of the BV2 microglial group III mGluR was attenuated by p44/42ERK and p38MAPK inhibition (Fig. 4.7Ci). However, in primary microglia L-AP4 induced dHEth fluorescence could only be attenuated by co-treatment with apocynin and the p38MAPK inhibitor (Fig. 4.7Cii), which corresponded with the NADPH oxidase activity assay data (Fig. 4.7Ciii). NADPH oxidase activation and superoxide production induced by activation of the group III mGluRs is therefore mediated by p38MAPK activation.

The involvement of p38MAPK and p44/42ERK in $GABA_A$ and P2Y2/4 receptor induced superoxide production was next investigated (Fig. 4.8). As before, primary and BV2

microglia were treated with the $GABA_A$ receptor agonist muscimol (50 μM) (Fig. 4.8Ai, ii, iii), or the P2Y2/4 receptor agonist UTPγS (100 μM) (Fig. 4.8Bi, ii, iii) in the presence or absence of apocynin (10 μM), the p44/42ERK inhibitor PD-98059 (PD, 30 μM), or the p38MAPK inhibitor SB-203580 (SB, 10 μM). Superoxide production was assessed by NBT reduction in BV2 microglia (Fig. 4.8Ai, Bi), dHEth fluorescence microscopy in primary microglia (Fig. 4.8Aii, Bii), whilst NADPH oxidase activity was assessed by the NADPH oxidase activity assay in BV2 microglia (Fig. 4.8Aiii, Biii).

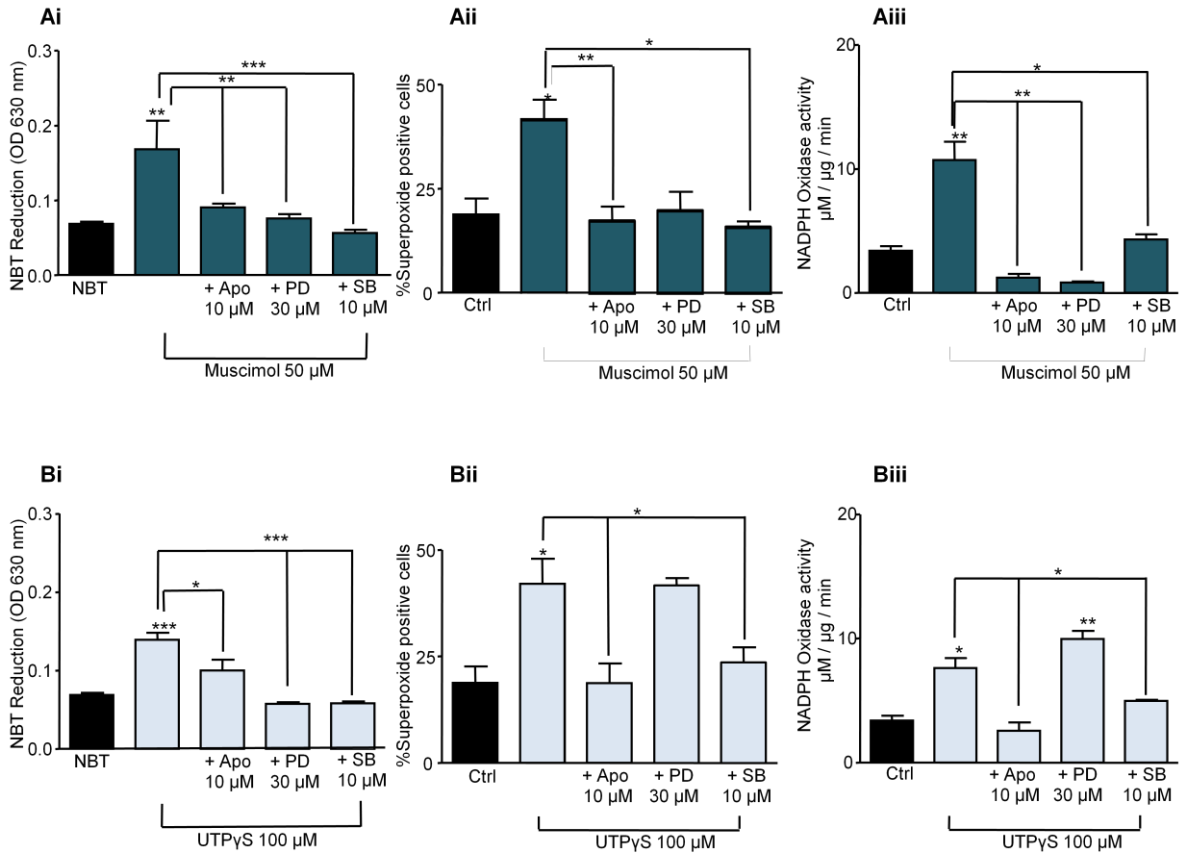

Figure 4.8 Inhibition of p44/42ERK or p38MAPK attenuates superoxide production and NADPH oxidase activity following modulation of the $GABA_A$ receptor or the P2Y2/4 receptor. *Primary and BV2 microglia were treated with the $GABA_A$ receptor agonist muscimol (50 µM)* ***(Ai, ii, iii)****, or the P2Y2/4 receptor agonist UTPγS (100 µM)* ***(Bi, ii, iii)*** *in the presence or absence of apocynin (10 µM), the p44/42ERK inhibitor PD-90859 (PD, 30 µM), or the p38MAPK inhibitor SB-203580 (SB, 10 µM). Superoxide production was measured by NBT reduction following treatment of BV2 microglia for 4 h as described* ***(Ai, Bi)****, and dHEth fluorescence was used to analyse superoxide production in primary microglia after treatment for 24 h* ***(Aii, Bii),*** *whilst NADPH oxidase activity was analysed in BV2 microglia after 4 h incubation, using the NADPH oxidase activity assay* ***(Aiii, Biii).*** *All data were analysed by one way ANOVA with Tukey post-hoc analysis, and comparisons were made between untreated controls and treatments in the presence of inhibitors as indicated. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

Muscimol induced NBT reduction in BV2 microglia was attenuated by co-treatment with the p44/42ERK or the p38MAPK inhibitor (Fig. 4.8Ai), which correlated with the NADPH oxidase activity data (Fig. 4.8Aiii). However, the increase in dHEth fluorescence following activation of the microglial $GABA_A$ receptor with muscimol could only be attenuated by p38MAPK inhibition (Fig. 4.8Aii). In BV2 microglia, activation of the $GABA_A$ receptor therefore induces superoxide production in a p38MAPK and p44/42ERK dependent manner, whereas in primary microglia, only p38MAPK is implicated in $GABA_A$ receptor induced NADPH oxidase activation.

UTPγS significantly elevated NBT reduction in BV2 microglia, which was attenuated by p38MAPK and p44/42ERK inhibition (Fig. 4.8Bi). Activation of the P2Y2/4 receptor in primary microglia significantly increased dHEth fluorescence, which was attenuated by co-treatment with the p38MAPK inhibitor (Fig. 4.8Bii). P2Y2/4 induced NADPH oxidase activity was also attenuated by p38MAPK inhibition (Fig. 4.8Biii), whereas inhibition of p44/42ERK increased P2Y2/4 receptor induced NADPH oxidase activity when compared with untreated cells, which may suggest a mechanism by which p44/42ERK negatively modulates P2Y2/4 receptor mediated superoxide production. These data show that p38MAPK activation regulates P2Y2/4 receptor induced NADPH oxidase activation and superoxide production.

The involvement of p38MAPK and p44/42ERK in iGluR induced superoxide production was also investigated. BV2 or primary microglia were treated with NAAG (100 µM) or MK-801 (10 µM) (Fig. 4.9Ai, ii), or QA (10 µM) or CNQX (10 µM) (Fig. 4.9Bi, ii), in the presence or absence of apocynin (10 µM), PD-98059 (30 µM) or SB-203580 (10µM). Superoxide production was assessed in BV2 microglia using the NBT assay (Fig. 4.9 Ai, Bi), and in primary microglia using dHEth fluorescence miscroscopy (Fig. 4.9 Aii, Bii).

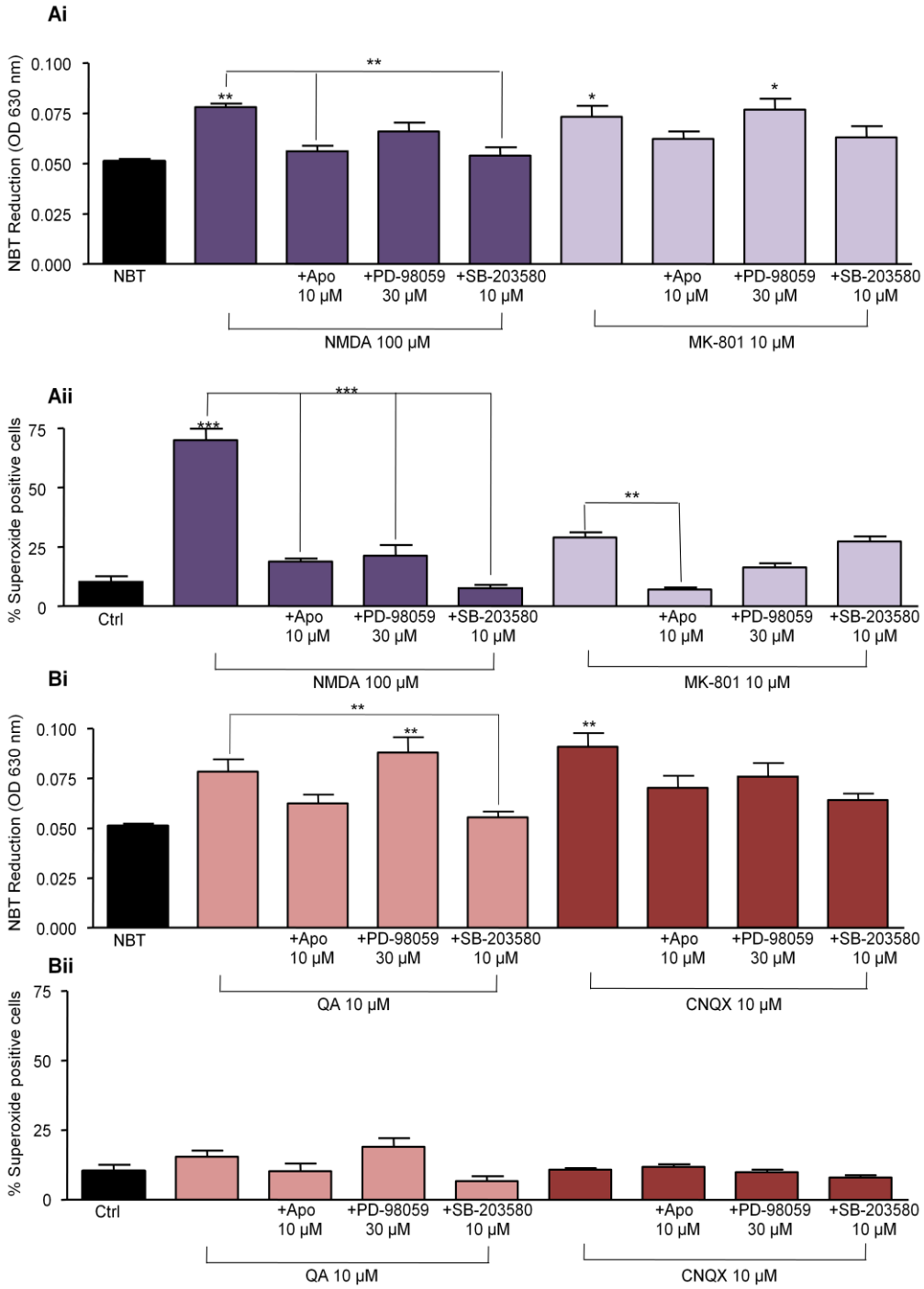

Figure 4.9 Inhibition of p44/42ERK or p38MAPK attenuates superoxide production and NADPH oxidase activity following modulation of iGluR's. *Primary and BV2 microglia were treated with the NMDA receptor agonist NMDA (100 µM)* ***(Ai, ii)****, or the NMDA receptor antagonist MK-801(10 µM)* ***(Ai, ii),*** *or the AMPA receptor agonist QA (10 µM)* ***(Bi, ii)*** *or the antagonist CNQX (10 µM)* ***(Bi, ii)*** *in the presence or absence of apocynin (10 µM), the p44/42ERK inhibitor PD-90859 (30 µM), or the p38MAPK inhibitor SB-203580 (10 µM). Superoxide production was assessed in BV2 microglia using the NBT assay* ***(Ai, Bi)*** *following treatment for 4 h. Superoxide production was analysed in primary microglia after treatment for 24 h, using dHEth fluorescence and imaging of superoxide positive cells* ***(Aii, Bii).*** *All data were analysed by one way ANOVA with Tukey post-hoc analysis and comparisons were made between control untreated cells and treatments, and also as indicated. *p<0.05, **p<0.01, ***p<0.001.Data are n=3.*

Activation of the BV2 microglial NMDA receptor significantly elevated NBT reduction that could be attenuated by p38MAPK inhibition, but not by inhibition of p44/42ERK (Fig. 4.9Ai). Induction of superoxide production through inhibition of the NMDA receptor with MK-801 in BV2 microglia could not be attenuated by apocynin, PD-98059 or SB-203580 (Fig. 4.9Ai), suggesting that superoxide production induced by inhibition of the NMDA receptor is through the activation of another oxidase system. In primary microglia (Fig. 4.9Aii), NMDA induced superoxide production was attenuated by p38MAPK or p44/42ERK inhibition, whereas MK-801 induced dHEth fluorescence could only be attenuated by co-treatment with apocynin (Fig. 4.9Aii). In BV2 microglia therefore, NMDA receptor induced superoxide production is mediated by p38MAPK signalling, whereas in primary microglia, both p38MAPK and p44/42ERK activation are implicated in NADPH oxidase activation.

Activation of the BV2 microglial AMPA receptor with QA did not significantly elevate NBT reduction (Fig. 4.9Bi), as shown previously. Inhibition of the AMPA receptor with CNQX increased NBT reduction; however this could not be attenuated by any of the inhibitors (Fig. 4.9Bi). Treatment of primary microglia with the AMPA receptor agonist QA or the antagonist CNQX could not increase dHEth fluorescence, and attenuation of the p38MAPK or p44/42ERK pathways had no effect on superoxide production.

It has been suggested that in addition to activation of p38MAPK or p44/42ERK inducing NADPH oxidase activation and subsequent superoxide production, superoxide production itself can modulate the phosphorylation of these signalling molecules (Sun et al. 2008), which may in turn perpetuate NADPH oxidase activity and superoxide production. To determine whether neurotransmitter receptor induced superoxide production could modulate p38MAPK or p44/42ERK phosphorylation, primary microglia were treated with the mGluR group III agonist L-AP4 (100 μM), the mGluR group I antagonist MTEP (100 nM), the mGluR3

agonist NAAG (50 μM), the $GABA_A$ receptor agonist muscimol (50 μM) or the P2Y2/4 receptor agonist UTPγS (100 μM) in the presence or absence of apocynin (10 μM) for 24 h. Microglia were then lysed, and lysates were subjected to Western blot analysis for phospho-p38MAPK (Fig. 4.10Ai, ii) or phospho-p44/42ERK (Fig. 4.10Bi, ii) expression. Western blots were run three times, using lysates from three separate microglial preparations. Expression of phospho-p38MAPK was normalised to total p83MAPK expression (Fig. 4.10Aii), and expression of phospho-p44/42ERK was normalised to β-actin (Fig. 4.10Bii).

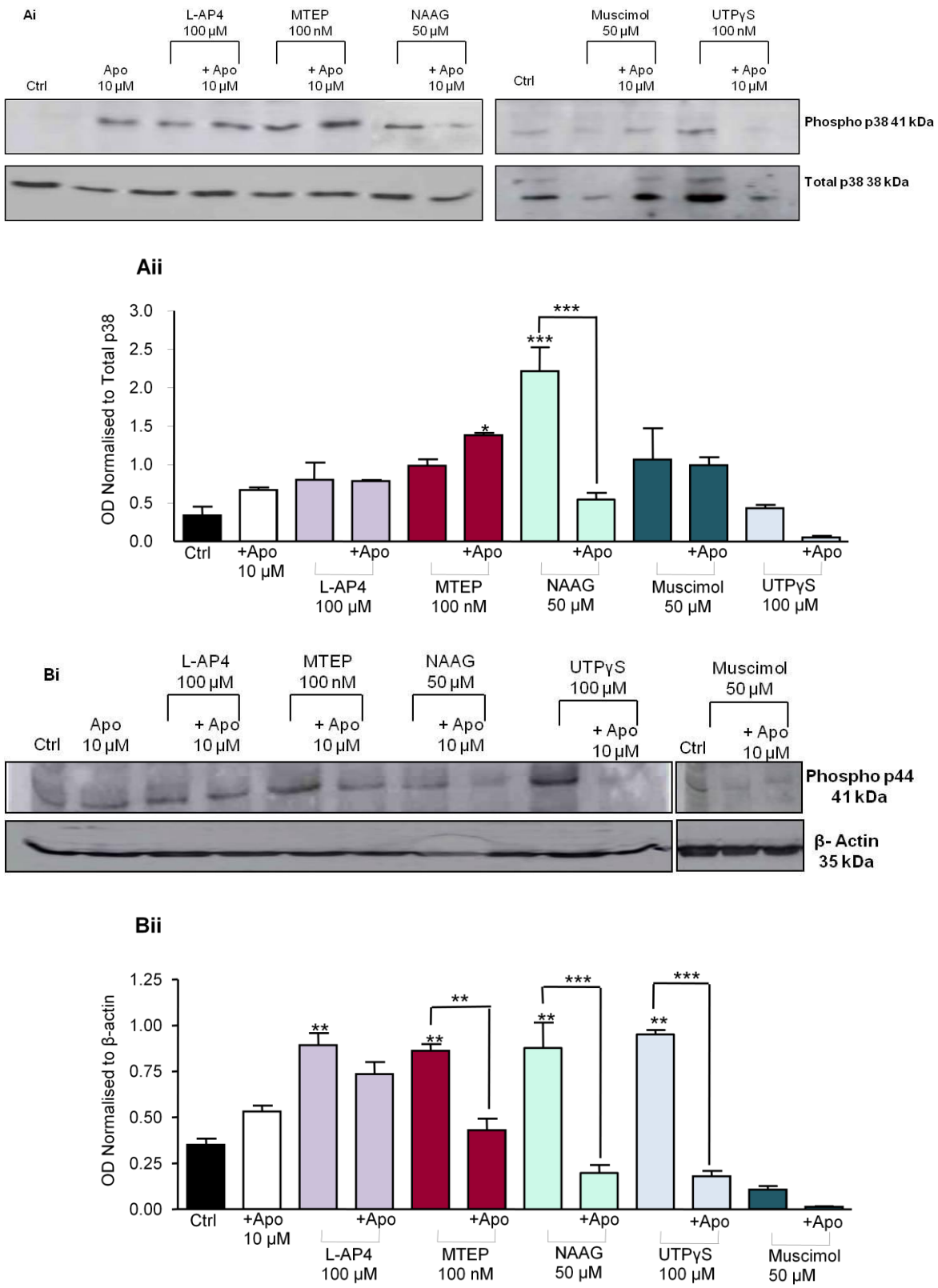

Figure 4.10 legend overleaf

Figure 4.10 Neurotransmitter receptor induced superoxide production modulates p38MAPK and p44/42ERK phosphorylation. *Primary microglia were treated with the mGluR group III agonist L-AP4 (100 µM), the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 µM), the $GABA_A$ receptor agonist muscimol (50 µM) or the P2Y2/4 receptor agonist UTPγS (100 µM) in the presence or absence of apocynin (10 µM) for 24 h. Microglia were lysed and Western blotting was performed using 45 µg cell lysate. After transfer to a PVDF membrane, membranes were probed for phospho-p38MAPK using a rabbit-anti-phospho-p38MAPK(T180)antibody (1:1000, 4°C, overnight), followed by an anti-rabbit-HRP secondary antibody (1:2000, 1 h room temperature) and visualisation was by ECL (**Ai).** The membrane was stripped and re-probed with an anti-rabbit-total p38MAPK antibody (1:1000, 4 °C, overnight), before incubation with the aforementioned secondary antibody and visualisation by ECL **(Ai).** The Western blots were performed three times to enable semi-quantitative expression analysis by densitometry **(Aii).** Optical density of phospho-p38MAPK was normalised to total p38MAPK for each condition to show expression levels **(Ai).** Lysates were also analysed for p44/42ERK expression, in which membranes were incubated with an anti-rabbit-p44/42ERK antibody (1:1000, 4°C, overnight), followed by incubation with the anti-rabbit-HRP secondary antibody (1:2000, 1 h , room temperature) and visualisation by ECL **(Bi)**. Membranes were then stripped and incubated with mouse-anti-β-actin antibody (1:2000, 2 h room temperature), and were then incubated with the anti-mouse-HRP secondary antibody (1:1000, 1 h room temperature) before visualisation by ECL **(Bi)**. The Western blots were again performed three times using lysates from three separate microglial preparations to enable semi-quantitative analysis of phospho-p44/42ERK expression, which was determined by densitometry **(Bii).** Data were analysed by one way ANOVA and Tukey post hoc analysis where comparisons were made between treatments and control un-treated cells, and also between conditions as indicated. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

Treatment of primary microglia with NAAG significantly elevated phospho-p38MAPK expression when compared with control untreated cells, which was significantly decreased upon inhibition of NADPH oxidase derived superoxide production with apocynin (Fig. 4.10Ai, ii), suggesting that mGluR3 induced superoxide production mediates p38MAPK activation. Treatment of microglia with the mGluR group I antagonist MTEP and apocynin significantly increased p38MAPK phosphorylation when compared with control untreated microglia (Fig. 4.10Ai, ii), therefore suggesting that attenuation of MTEP induced NADPH oxidase activation mediates p38MAPK phosphorylation.

Treatment of primary microglia with MTEP, NAAG or UPTγS significantly elevated p44ERK phosphorylation in an NADPH oxidase dependent manner, as phosphorylation was significantly reduced upon co-treatment with apocynin (Fig. 4.10Bi, ii). Activation of the group III mGluRs with L-AP4 significantly enhanced p44/42ERK phosphorylation when compared with control untreated cells; however, this phosphorylation was not dependent on activation of the NADPH oxidase.

The phosphorylation of p38MAPK was next investigated after modulation of iGluRs. Primary microglia were treated with NMDA (100 μM) or MK-801 (10 μM) alone, in the presence of apocynin (10 μM), or in combination for 24 h. Western blotting for phospho-p38MAPK was then performed (Fig. 4.11Ai,ii) using three separate microglial preparations to enable densitometry to be performed (Fig. 4.11Aii). Microglia were also treated with the AMPA receptor agonist QA (10 μM), the antagonist CNQX (10 μM) either alone, in combination, or in the presence of apocynin (10 μM) for 24 h before Western blot analysis for phospho-p38MAPK expression (Fig. 4.11Bi, ii), and densitomety (Fig. 4.11Bii) were performed.

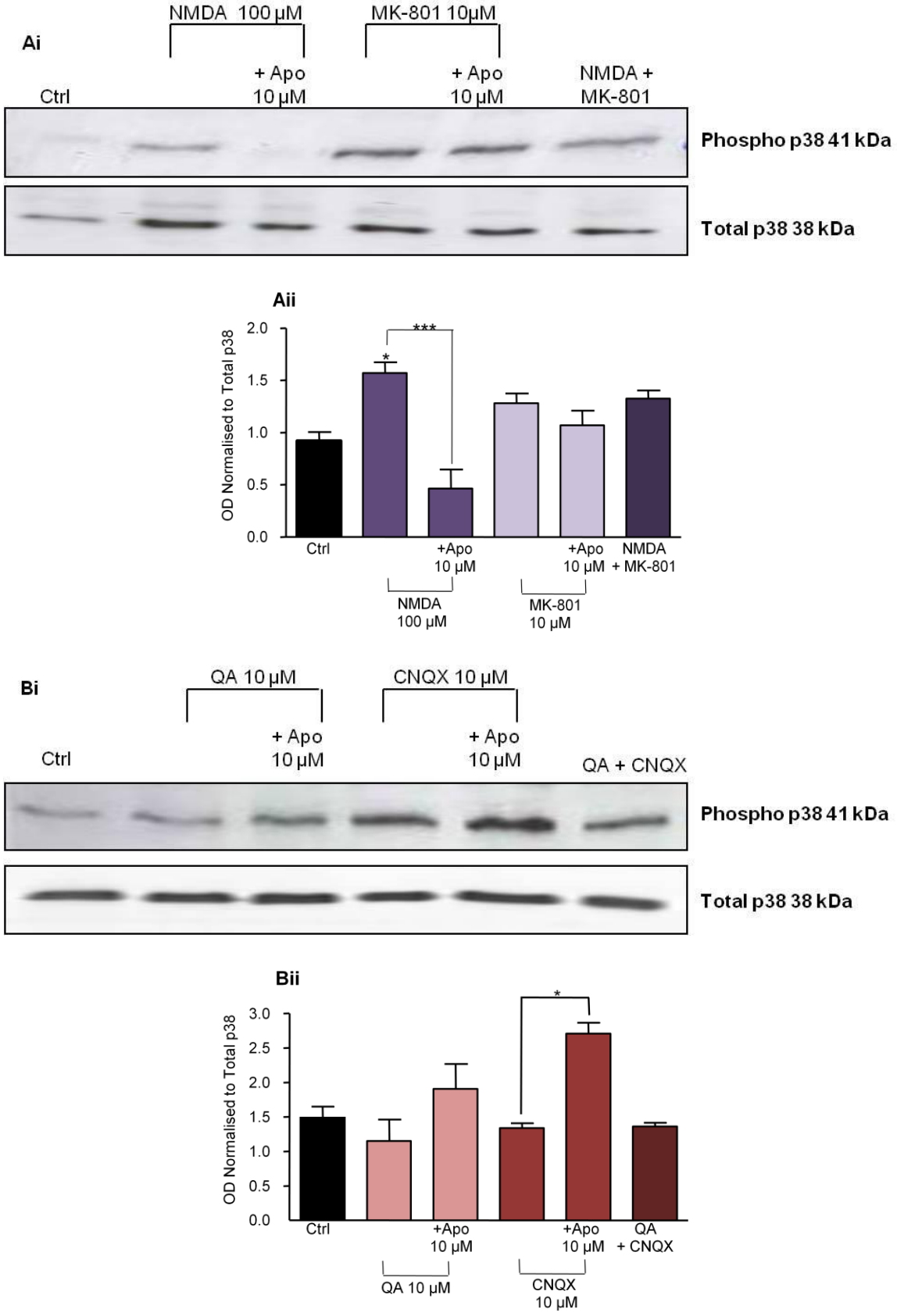

Figure 4.11 legend overleaf

Figure 4.11 iGluR induced superoxide production modulates p38MAPK phosphorylation. *Primary microglia were treated with the NMDA receptor agonist NMDA (100 µM) or the NMDA receptor antagonist MK-801 (10 µM) either alone, in combination or with apocynin (10 µM) for 24 h* ***(Ai, ii).*** *Microglia were also treated with the AMPA receptor agonist QA (10 µM), or the AMPA receptor antagonist CNQX (10 µM) either alone, in combination or in the presence of apocynin (10 µM) for 24 h* ***(Bi, ii)****. Microglia were lysed and Western blotting was performed using 45 µg cell lysate. After transfer to a PVDF membrane, membranes were probed for phospho-p38MAPK using a rabbit-anti-phospho-p38MAPK(T180) antibody (1:1000, 4°C, overnight), followed by incubation with an anti-rabbit-HRP secondary antibody (1:2000, 1 h room temperature) and visualisation by ECL (****Ai, Bi).*** *The membrane was stripped and re-probed with an anti-rabbit-total p38MAPK antibody (1:1000, 4 °C, overnight), before incubation with the aforementioned secondary antibody and visualisation by ECL* ***(Ai, Bi).*** *The Western blots were performed three times to enable semi-quantitative expression analysis by densitometry. Optical density of phospho-p38MAPK was normalised to total p38MAP for each condition to show expression levels* ***(Aii, Bii).*** *Data were analysed by one way ANOVA and Tukey post-hoc analysis. *p<0.05, **p<0.001. All data are n=3.*

NMDA significantly increased p38MAPK phosphorylation in comparison with control untreated cells, which was attenuated by co-treatment of microglia with NMDA and apocynin (Fig. 4.11Ai, ii), suggesting that NMDA receptor induced activation of the NADPH oxidase and subsequent superoxide production regulates p38MAPK phosphorylation. Treatment of primary microglia with the AMPA receptor agonist QA or the antagonist CNQX did not significantly modulate p38MAPK phosphorylation, however co-treatment of microglia with CNQX and apocynin significantly elevated p38MAPK phosphorylation when compared with control or CNQX treatments alone (Fig. 4.11Bi, ii) therefore suggesting that inhibition of the NADPH oxidase after inhibition of the AMPA receptor mediates p38MAPK activation.

Microglial p44/42ERK phosphorylation was next investigated after iGluR modulation. Primary microglia were treated with the NMDA receptor agonist NMDA (100 μM), the antagonist MK-801 (10 μM) either alone, in combination or in the presence of apocynin (10 μM); or the AMPA receptor agonist QA (10 μM), the antagonist CNQX (10 μM) either alone, in combination, or with apocynin (10 μM) for 24 h before lysis. Lysates were subjected to Western blotting for p44/42ERK phosphorylation (Fig. 4.12A), and densitometry was performed following normalisation of protein levels to β-actin (Fig. 4.12B).

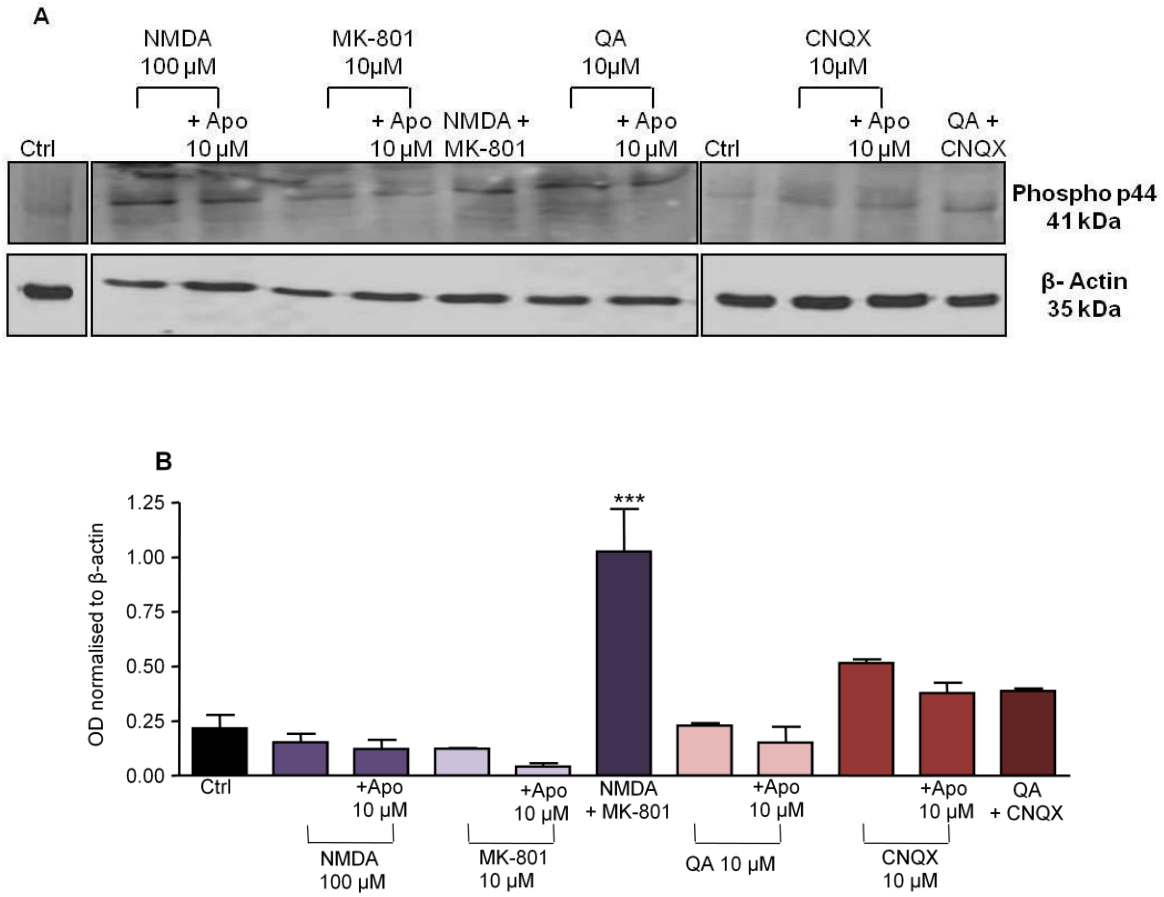

Figure 4.12 iGluR induced superoxide production modulates p44/42ERK phosphorylation. *Primary microglia were treated with the NMDA receptor agonist NMDA (100 µM) or the NMDA receptor antagonist MK-801 (10 µM) either alone, in combination or with apocynin (10 µM) for 24 h, or with the AMPA receptor agonist QA (10 µM), or the AMPA receptor antagonist CNQX (10 µM) either alone, in combination or in the presence of apocynin (10 µM) for 24 h.. Microglia were lysed and Western blotting was performed using 45 µg cell lysate. After transfer to a PVDF membrane, membranes were probed for phospho-p44/42ERK using a rabbit-anti-phospho-p44/42ERK antibody (1:1000, 4°C, overnight), followed by an anti-rabbit-HRP secondary antibody (1:2000, 1 h room temperature) and visualisation was by ECL* ***(A).*** *The membrane was stripped and re-probed with a mouse-anti-β-actin antibody (1:2000, 2 h room temperature), before incubation with an anti mouse HRP secondary antibody (1:1000, 1h, room temperature) and visualisation was by ECL* ***(A).*** *The Western blots were performed three times to enable semi-quantitative expression analysis by densitometry. Optical density of phospho-p44/42ERK was normalised to β-actin for each condition to quantify expression levels* ***(B).*** *Data were analysed by one way ANOVA with Tukey post-hoc analysis. ***p<0.001. All data are n=3.*

Modulation of the microglial iGluRs had no effect on p44/42ERK phosphorylation (Fig. 4.12); however, co-treatment of microglia with the NMDA receptor agonist and antagonist significantly elevated p44/42ERK phosphorylation which was not a consequence of NADPH oxidase activation. Modulation of the NMDA receptor and induction of superoxide production therefore elevates p38MAPK phosphorylation rather than p44/42ERK phosphorylation.

Together, these data show that there may be a feedback activation mechanism whereby superoxide production following receptor modulation may induce p38MAPK or p44/42ERK activation, and that this p38MAPK or p44/42ERK activation may regulate NADPH oxidase activity and superoxide production.

4.2.4 Microglial activation is not modulated by neurotransmitter induced superoxide production

Modulation of the microglial p44/42ERK and p38MAPK pathways can induce a reactive microglial phenotype. To investigate whether neurotransmitter and receptor modulation, shown to induce NADPH oxidase activity through activation of p44/42ERK and p38MAPK, could modulate the activation state of microglia, immunocytochemisty for the activation markers iNOS or ED1, was performed. Western blotting for iNOS was also conducted.

Primary microglia were treated with the positive control for microglial activation, LPS (1 µg/ml), or the positive control for NADPH oxidase activation, PMA (10 ng/ml), and the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) for 24 h before immunocytochemistry was performed using antibodies for iNOS (red) or ED1 (green) (Fig. 4.13A). To investigate microglial reactivity further, Western blotting for iNOS expression was also performed on primary microglia treated as above for 24 h, and β-actin expression was used as a loading control (Fig. 4.13B).

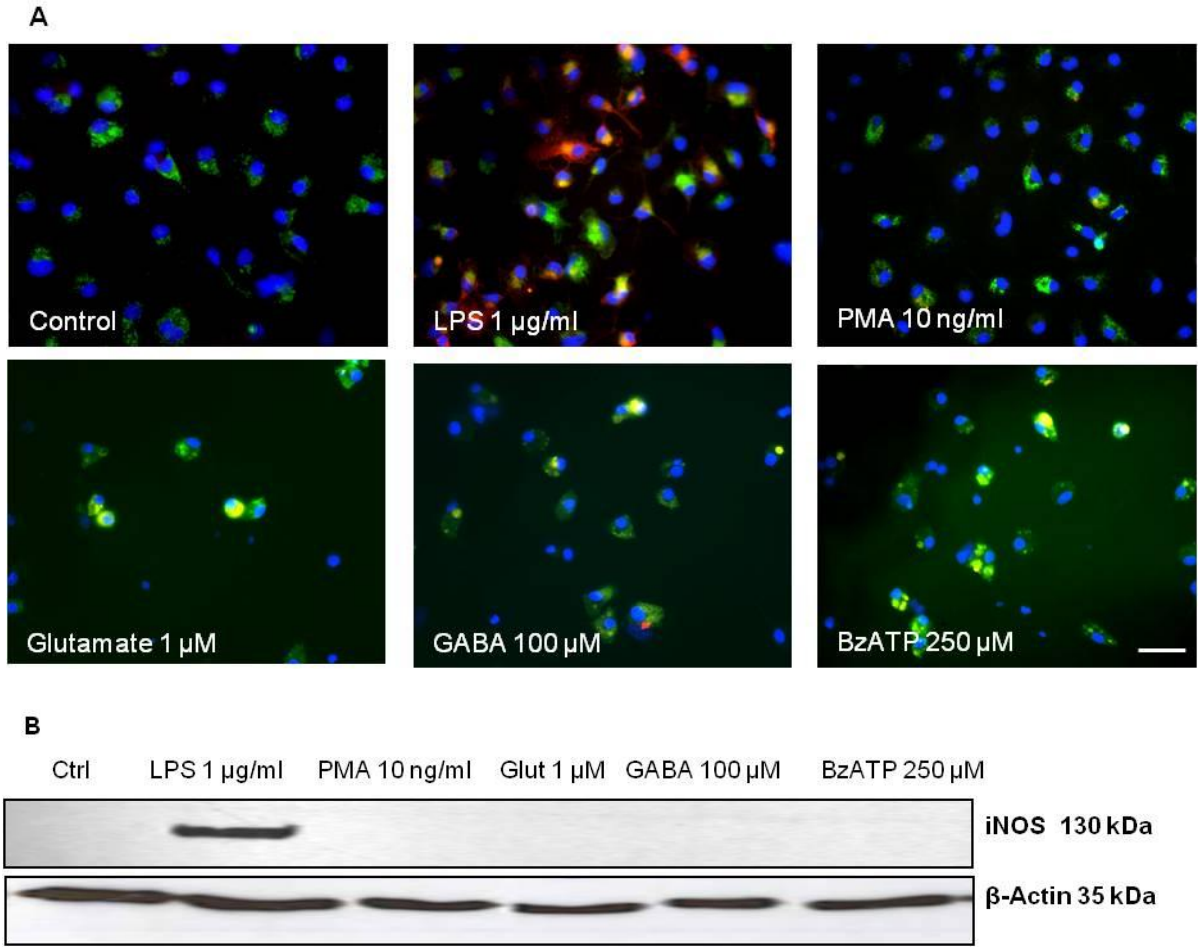

Figure 4.13 Modulation of microglial reactivity after treatment with neurotransmitters. *Microglia were treated with the positive control LPS (1 µg/ml), PMA (10 ng/ml), or the neurotransmitters glutamate (1 µM), GABA (100 µM), or BzATP (250 µM) for 24 h. Cells were then either fixed for immunocytochemistry* ***(A)****, or lysed for Western blotting* ***(B).*** *Immunocytochemistry was performed using a mouse-anti-ED1 antibody (1:100) (Green) and a rabbit-anti-iNOS antibody (1:500) (Red). Cells were incubated with the primary antibodies overnight before incubation with the secondary antibodies anti-mouse FITC antibody (1:1000) for ED1, and anti-rabbit TRITC antibody (1:1000) for iNOS. Cells were counterstained with DAPI (1:2000) and were imaged using by fluorescence microscopy with a 40x objective* ***(A).*** *Microglia were lysed in lysis buffer for Western blot analysis, and 45 µg of protein was run on a 10% SDS-PAGE gel before transfer to a PVDF membrane. After blocking, the membrane was incubated with the rabbit- anti-iNOS antibody(1:5000) for 2 h at room temperature, then with the anti-rabbit-HRP secondary antibody (1:2000) for 1 h at room temperature. Visualisation was by ECL. The membrane was then stripped and re-probed with the mouse-anti-β-actin antibody (1:2000) for 2 h at room temperature, and then the anti-mouse HRP secondary antibody (1:1000) for 1 h at room temperature, Visualisation was by ECL* ***(B).*** *Both experiments are n=3. Scale bar 20 µm.*

The immunocytochemistry (Fig. 4.13A) and Western blotting (Fig. 4.13B) data show that treatment of primary microglia with LPS (1 μg/ml) elevates iNOS expression in comparison to control untreated cells and also when compared with PMA, glutamate, GABA or BzATP treatments, which could not induce iNOS expression in primary microglia. The marker for phagocytosis (ED1) was up-regulated in microglia treated with LPS, glutamate or BzATP (Fig. 4.13A), suggesting that these neurotransmitters induce a phagocytic phenotype. This may not however, be a fully active phenotype, as iNOS expression was not increased.

The effects of neurotransmitter receptor modulation on microglial reactivity was next investigated using Western blot analysis to detect iNOS expression in microglia treated with LPS (1 μg/ml), the mGluR group III agonist L-AP4 (100 μM), the mGluR3 agonist NAAG (50 μM), the mGluR group I antagonist MTEP (100 nM), the $GABA_A$ receptor agonist muscimol (50 μM), or the P2Y2/4 receptor agonist UTPγ (100 μM) for 24 h (Fig. 4.14A). Western blotting was also performed for β-actin, to semi-quantify iNOS expression levels following normalisation to β-actin (Fig. 4.14B).

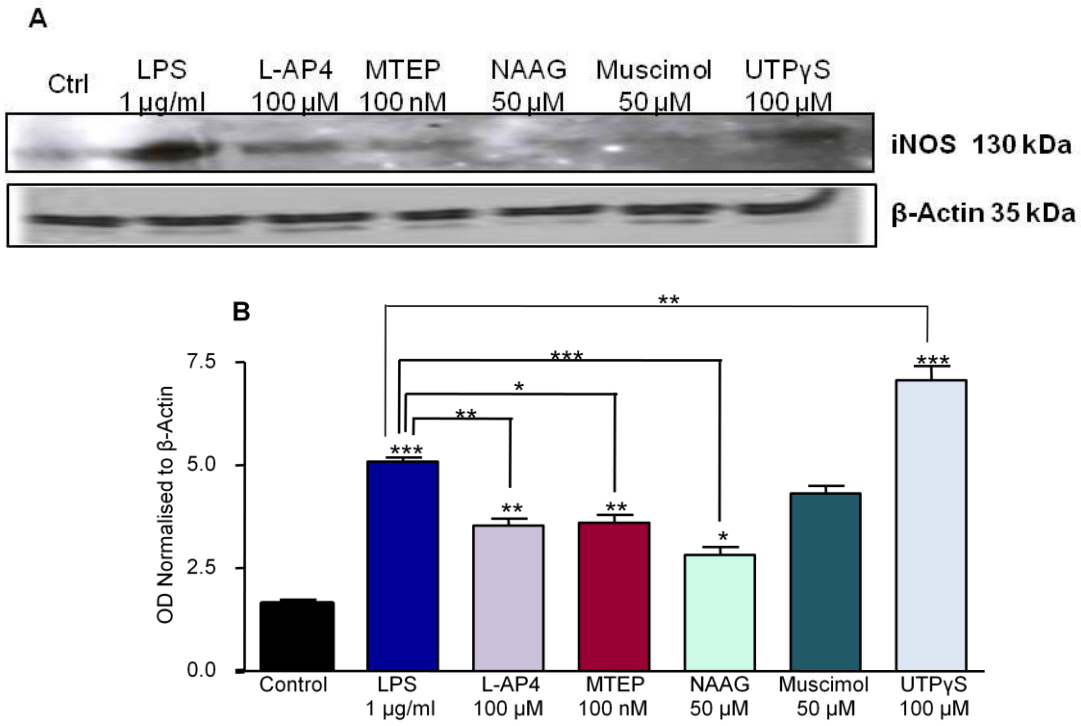

Figure 4.14 Microglial recactivity following modulation of neurotransmitter receptors. *Microglia were treated with the microglial activator LPS (1 µg/ml), the mGluR group III agonist L-AP4 (100 µl), the mGluR3 agonist NAAG (50 µM), the mGluR group I antagonist MTEP (100 nM), the $GABA_A$ receptor agonist muscimol (50 µM), or the P2Y2/4 receptor agonist UTPγ (100 µM) for 24 h. Microglia were lysed in lysis buffer for Western blot analysis, and 45 µg of protein was run on a 10% SDS-PAGE gel before transfer to a PVDF membrane. After blocking, the membrane was incubated with the rabbit- anti-iNOS antibody (1:5000) for 2 h at room temperature, then with the anti-rabbit-HRP secondary antibody (1:2000) for 1 h at room temperature. Visualisation was by ECL. The membrane was then stripped and re-probed with the mouse-anti-β-actin antibody for 2 h at room temperature, and then the anti-mouse HRP secondary for 1 h at room temperature. Visualisation was by ECL* ***(A).*** *Densitometry was performed to determine iNOS expression levels normalised to β-actin* ***(B).*** *The experiment was performed three times, and statistical analysis on the densitomety was performed using a one way ANOVA with Tukey post-hoc analysis, in which comparisons were made between all conditions and the control cells, and as inducated. *p<0.05, **p<0.01, ***p<0.001. Data are n=3.*

Treatment of microglia with the mGluR group I receptor antagonist MTEP, the mGluR3 agonist NAAG and the group III mGluR agonist L-AP4 all induced a significant increase in iNOS expression when compared with control untreated cells (Fig. 4.14), however, LPS induced iNOS expression was significantly elevated in comparison with these treatments. Activation of the $GABA_A$ receptor with muscimol did not induce a significant increase in iNOS expression, whilst activation of the P2Y2/4 receptor induced an increase in iNOS expression comparable to that of LPS. This therefore suggests that modulation of mGluRs and the P2Y2/4 receptor induces a reactive phenotype, but that activation of the $GABA_A$ receptor may not.

These data could also suggest that neurotransmitter receptor mediated superoxide production, shown to be modulated by the MAPK signalling pathway, may be a consequence of microglial activation, and could represent a neuroprotective or neurotoxic phenotype, which will be investigated in the next chapter.

4.3 Discussion

Here, modulation of microglial neurotransmitter receptors altered Nox1, Nox2 or Nox4 expression and activity, which are regulated by PKCδ or PI3-K signalling. Furthermore, the MAPK signalling pathway was also shown to regulate neurotransmitter receptor induced superoxide production, and superoxide production itself could also modulate the phosphorylation of MAPK signalling molecules. The microglial NADPH oxidase and MAPK signalling cascade are implicated in the progression of neurodegenerative conditions, and the findings presented here showing cross-talk between NADPH oxidase activation and MAPK signalling could suggest that the activation or inhibition of microglial neurotransmitter receptors may exacerbate or protect against inflammatory neurodegenerative conditions.

4.3.1 Neurotransmitter and receptor induced NADPH oxidase isoform expression and activity in microglia

As described previously, microglia express Nox1 (Chéret et al. 2008), Nox2 (Wilkinson et al. 2006) and Nox4 (Harrigan et al. 2008) NADPH oxidase isoforms. Following the findings in the previous chapter that neurotransmitters or receptor agonists or antagonists induced microglial superoxide production, it was important to determine which NADPH oxidase isoforms were induced as a consequence of this receptor modulation, as this could have important consequences for microglial reactivity.

Nox4 expression was significantly enhanced by glutamate, GABA or BzATP. Nox4 is predominantly regulated at the transcriptional level (Manea et al. 2010), as it lacks the cytoplasmic subunits that are required for Nox1 and Nox2 activation (Brown & Griendling 2009). Nox4 transcription can be induced by NF-κB, and also by JAK/STAT signalling, which promotes the binding of the STAT1/2 transcription factor to specific regulation sequences in the Nox4 promotor, known as GAS elements or ISRE's (Manea et al. 2010) which induce the expression of redox regulated genes (Wesoly et al. 2007). In a human microglial cell line, Nox4 expression is induced by IFNγ (Li et al. 2009), which activates STAT1/2 and facilitates Nox4 transcription through activation of the GAS element (Manea et al. 2010). Glutamate, GABA or BzATP may therefore induce the translocation of transcription factors such as NF-κB or STAT1/2 to the nucleus, resulting in microglial Nox4 expression.

Nox1 is regulated by phosphorylation induced translocation of cytoplasmic subunits and also at the transcriptional level (Fan et al. 2005; Wei et al. 2010), and GABA and BzATP both induced Nox1 transcription. The transcriptional regulation of Nox1 has been demonstrated in VSMC's, in which Nox1 expression was induced by TNFα mediated activation of NF-κB (Manea et al. 2010). In addition, TNFα binding to TNFR1 mediates the binding of Nox1 to

TNFR1 associated death domain (TRADD), which promotes ROS induced JNK activation and perpetuates superoxide production in endothelial cells (Kim et al. 2007). Furthermore, TNFα can induce sustained activation of p38MAPK and JNK, which enhances the phosphorylation of the transcription factors c-Jun and c-Fos, resulting in further Nox1 transcription and enhanced superoxide production (Kuwano et al. 2008). Treatment of microglia with BzATP promotes TNFα release (Suzuki et al. 2004), which may therefore feedback onto microglia to enhance Nox1 expression and activation (Mir et al. 2009).

In cardiovascular tissue Nox1 expression is induced by the transcription factor c-Jun, which is activated by p44/42ERK phosphorylation (Cevik et al. 2008), and Nox1 expression is also down-regulated in adenocarcinoma cells following inhibition of p44/42ERK (Adachi et al. 2008). In microglia, activation of p44/42ERK induces NADPH oxidase activity after exposure to paraquat (Miller et al. 2007) or LPS (Zhao et al. 2011), suggesting a role for p44/42ERK in microglial Nox1 activation. This could therefore imply that GABA or BzATP treatment promote Nox1 expression through p44/42ERK signalling in microglia.

In addition to regulating phosphorylation of the NADPH oxidase p47phox and p67phox subunits, PKCδ can induce Nox1 expression through p44/42ERK phosphorylation dependent activation of activating transcription factor 1 (ATF-1) and subsequent binding to the Nox1 promoter (Fan et al. 2005). GABA mediates PKCδ activation (Fukura et al. 1996) and modulates p44/42ERK activation (Bell-Horner et al. 2006), whilst P2X7 receptor activation mediates p44/42ERK phosphorylation (Takenouchi et al. 2007), which promotes Nox1 transcription, lending further support to the suggestion that GABA and BzATP enhance Nox1 expression.

Nox2 expression was not modulated by glutamate, GABA or BzATP, and is not thought to be controlled at the transcriptional level. Rather, modulation of Nox2 activity occurs through

the regulated phosphorylation of cytoplasmic subunits (El-Benna et al. 2009). The regulation of NADPH oxidase isoform activity through phosphorylation of cytoplasmic subunits was investigated using inhibitors of kinases known to induce subunit phosphorylation, along with the specific inhibitors apocynin, shown to inhibit p47phox phosphorylation, and therefore act on Nox1 and Nox2 (Stefanska & Pawliczak 2008); and thioridazine, a Nox4 inhibitor (Serrander et al. 2007; Harrigan et al. 2008). Nox1 activity was inhibited with the PKCδ inhibitor rottlerin (Miller et al. 2007), and the PI3-K inhibitor wortmannin was used to inhibit p40phox phosphorylation and Nox2 activity (Ellson et al. 2006).

Glutamate - induced superoxide production and NADPH oxidase activity was attenuated by wortmannin and rottlerin, suggesting that glutamate mediates Nox1 and Nox2 activation through PKCδ and PI3-K signalling. Glutamate mediates the PKC induced expression of neurotrophic factors in microglia (Liang et al. 2010), and in astrocytes, glutamate promotes PKCδ activation and translocation to the plasma membrane through Ca^{2+} signalling (Codazzi et al. 2001), which could suggest a mechanism whereby glutamate promotes PKC mediated Nox1/2 activation. Glutamate promotes PI3-K activity in glial cells during development, in which PI3-K activity promotes the mobilisation of NF-κB to the nucleus to induce transcription of trophic factors (Balazs 2006). NF-κB activation also promotes Nox1 expression (Manea et al. 2010), therefore glutamate induced PI3-K signalling may mediate Nox1 and Nox2 activation. Glutamate induces p38MAPK phosphorylation in Bergmann glial cells (Zepeda et al. 2008), which promotes Nox2 activity (Anilkumar et al. 2008). Furthermore, there may be cross-talk between p38MAPK and PI3-K, as it has been shown that NO can activate both pathways and induce superoxide production in a Nox2 dependent manner in VSMC's (Doronzo et al. 2011). Glutamate could therefore facilitate microglial Nox1 and Nox2 activation in this manner. Microglial glutamate induced superoxide production was also attenuated by thioridazine, suggesting that glutamate regulates Nox4

activity. In cortical neurons, glutamate induces H_2O_2 production in a Nox4 dependent manner (Ha et al. 2010), which may also occur in microglia, and supports the findings presented here.

GABA induced superoxide production and NADPH oxidase activity was attenuated by inhibitors of all three NADPH oxidase isoforms in BV2 microglia, however, in primary microglia, superoxide production could only be attenuated by co-treatment of cells with GABA and apocynin. Inhibition of GABA induced superoxide production by rottlerin in BV2 microglia suggests that GABA promotes PKCδ activity, which enhances Nox1 expression and activity (Fan et al. 2005). This also correlates with the expression analysis showing that GABA up-regulates Nox1 transcription. There is a close relationship between $GABA_A$ receptor activation and PKC phosphorylation, and PKC interacts with the $GABA_A$ receptor to regulate GABA binding (Moss et al. 1992). Furthermore, in neurons $GABA_A$ receptor activation induces PKC phosphorylation through PKC binding to the γ subunit of the receptor (Brandon et al. 1999). Exposure of microglia to GABA could therefore induce PKC activation which facilitates Nox1 activity and expression, and could perpetuate Nox1 and PKC activity through a feedback mechanism.

In primary microglia only treatment with the Nox1/2 inhibitor apocynin attenuated GABA induced superoxide production, therefore GABA-induced Nox1/2 activation was independent of PKCδ or PI3-K activation. The differences observed may be due to the cell types used. BV2 microglia may respond slightly differently to primary microglia, however studies have shown that BV2 microglia are a suitable substitute for primary microglia in a range of experimental protocols (Henn et al. 2009). Here therefore, GABA may elicit the activation of different signalling pathways in BV2 or primary microglia; however in both cell types GABA induces Nox1/2 activation.

BzATP induced superoxide production in BV2 microglia was attenuated by apocynin, thioridazine, rottlerin and wortmannin, however, in primary microglia only apocynin attenuated superoxide production. Co-treatment of primary microglia with BzATP and rottlerin or wortmannin significantly increased superoxide production when compared with control un-treated cells. Expression analysis showed that BzATP induced Nox1 and Nox4 transcription, which differs from the functional data. Together, the data show that BzATP induces superoxide production through Nox1 or Nox2 activation.

There are few reports regarding NADPH oxidase isoform activation by ATP, however, in macrophages, ATP stimulation induces Nox2 activity (Moore & MacKenzie 2009). Furthermore, ATP activation of macrophages elevates Nox2 dependent superoxide production through p38MAPK phosphorylation (Noguchi et al. 2008; Anilkumar et al. 2008). The increase in BzATP derived superoxide production following PKC and PI3-K inhibition in primary microglia could represent a feedback mechanism in which attenuation of superoxide production from specific Nox isoforms may enhance superoxide production through the activation of other Nox isoforms. In support of this suggestion, the redox state of embryonic stem cells regulates the transcription of NADPH oxidase isoforms, with mechanisms in place to enhance Nox1 and Nox4 transcription upon enhanced H_2O_2 production (Buggisch et al. 2007). Whilst it has not been shown that a deficit in superoxide production may enhance Nox isoform activity or expression, the presence of this feed-forward mechanism suggests that a regulatory mechanism may act to enhance Nox isoform activation upon inhibition of superoxide production.

Modulation of neurotransmitter receptors had little effect on NADPH oxidase isoform expression; however, modulation of microglial neurotransmitter receptors may have a greater effect on Nox isoform activity through the activation of signalling molecules known to induce phosphorylation of the cytoplasmic subunits of the NADPH oxidase.

Treatment of primary microglia with the mGluR group I antagonist MTEP did not increase NADPH oxidase expression, however, co-treatment of microglia with MTEP and the NADPH oxidase antagonist apocynin significantly elevated Nox1 and Nox4 expression when compared with control untreated cells. This could suggest that inhibition of Nox2 with apocynin may induce a feedback mechanism to enhance Nox1 and Nox4 expression, thereby regulating the intracellular redox potential.

MTEP induced superoxide production and NADPH oxidase activity in BV2 microglia was attenuated by all NADPH oxidase isoform inhibitors, therefore MTEP induces superoxide production through Nox1, Nox2 and Nox4 in a PKCδ dependent manner. In primary microglia however, MTEP induced superoxide production could only be attenuated by apocynin, and was independent of PI3-K or PKC signalling. Nox2 activation can be mediated by phospholipase-D (PLD) in neutrophils (Patel et al. 2010), and in the rat hippocampus, antagonism of the group I mGluRs mediates PLD signalling (Pellegrini-Giampietro et al. 1996), suggesting that in primary microglia, Nox2 activation following treatment with MTEP may be through PLD induction. Furthermore, PLD mediates Nox2 activation through p44/42ERK induction (Patel et al. 2010), and p44/42ERK inhibition was shown here to attenuate MTEP induced superoxide production in primary microglia, suggesting a novel mechanism of MTEP induced Nox2 activation which should be investigated further.

The finding that in primary microglia, MTEP induced superoxide production is not mediated by PKC signalling may be a consequence of the inhibitory action of mGluR group I antagonism on PKC activation, as it has been shown in spinal cord homogenates that group I mGluR inhibition attenuates PKC activity (Ferguson et al. 2008). Furthermore, activation of the group I mGluRs with DHPG increases PKC activation in glial cells (Peavy & Conn 1998), therefore here, inhibition of the group I mGluR may attenuate PKC signalling. The

data here therefore suggests that inhibition of the group I mGluRs with MTEP induces Nox1 and Nox2 mediated superoxide production, however a mechanism is still to be elucidated.

Activation of the microglial mGluR3 did not enhance NADPH oxidase isoform expression; however, Nox2 expression was up-regulated following treatment of microglia with NAAG and apocynin, suggesting the presence of a feedback mechanism in which inhibition of superoxide production increases Nox isoform expression. This is supported by studies in VSCM's in which attenuation of Nox1 induced superoxide production was shown to elevate Nox4 expression (Wosniak et al. 2009), which further supports suggestions that NADPH oxidase derived ROS are essential to normal cellular functions in the CNS (Infanger et al. 2006).

MGluR3 induced superoxide production was attenuated in BV2 microglia by all inhibitors, thereby suggesting that NAAG induces PKC and PI3-K activation. Activation of neuronal group II mGluRs elevates PKC activity, which also enhances NMDA receptor activation (Tyszkiewicz et al. 2004). Enhanced NMDA receptor activity also significantly elevated NADPH oxidase mediated superoxide production, which could suggest a mechanism whereby mGluR3 activation directly induces NADPH oxidase derived superoxide production and enhances NMDA receptor mediated NADPH oxidase activation in a PKC dependent manner. In addition, activation of mGluR3 in astrocytes induces PI3-K activity (Ciccarelli et al. 2007), which mediates Nox2 activation (Ellson et al. 2006). These studies therefore lend support to the findings that activation of mGluR3 in BV2 microglia induces Nox2 activity.

In primary microglia, mGluR3 mediated superoxide production was only attenuated by apocynin, thereby suggesting that mGluR3 induced Nox2 activation is independent of PI3-K or PKC signalling. Inhibition of PKC with rottlerin significantly enhanced NAAG induced superoxide production, lending support to the suggestion of a feedback mechanism in which

decreased superoxide production through inhibition of one Nox isoform may enhance the production of superoxide through promoting the activity of other Nox isoforms. This is supported by findings that in gp91phox (Nox2) knockout mice, Nox4 activity is enhanced after ischaemia, indicating a compensatory effect for superoxide production (Byrne et al. 2003).

Another mechanism could be suggested for mGluR3 mediated NADPH oxidase activation in primary microglia. Microglial mGluR3 activation is negatively coupled to adenlylate cyclase (Prézeau et al. 1994), and activation of adenylate cyclase with pituitary adenylate cyclase activating polypeptide (PACAP) inhibits microglial NADPH oxidase activity resulting in neuroprotection after LPS induced superoxide production, which can protect against dopaminergic neurotoxicity in mixed neuronal glial mesencephalic cultures (Yang et al. 2006). The inhibition of adenylate cyclase after activation of mGluR3 in primary microglia may induce NADPH oxidase activation, which is supported by findings that inhibition of adenylate cyclase stimulation following treatment of microglia with the inhibitor PACAP6-38 could not induce neuroprotection, and enhanced NADPH oxidase activity (Yang et al. 2006). Superoxide production through microglial mGluR3 activation could therefore be mediated through enhanced NMDA receptor activation (Tyszkiewicz et al. 2004) or through negative coupling to adenylate cylcase (Yang et al. 2006) in primary microglia, whereas in BV2 microglia, mGluR3 induced Nox2 activation may be a consequence of PI3-K and PKC signalling.

Activation of the group III mGluRs with L-AP4 did not modulate NADPH oxidase isoform expression, however L-AP4 induced superoxide production and NADPH oxidase activity in BV2 microglia was attenuated by treatment with apocynin, thioridazine or wortmannin, suggesting Nox2 and Nox4 activation. In primary microglia, L-AP4 induced superoxide production was only attenuated by apocynin, suggesting that Nox2 activation is independent

of PKC and PI3-K signalling. Activation of neuronal group III mGluRs does not induce PKC signalling, rather elicits down-stream effects through attenuating cyclic adenosine monophosphate (cAMP) signalling (Martín et al. 2007). Studies in VSMC's have shown that attenuation of cAMP elevates oxidative stress in an NADPH oxidase dependent manner (Saha et al. 2008). Furthernore, attenuation of cAMP elevated PMA induced -NADPH oxidase dependent superoxide production in neutrophils (Mitsuyama et al. 1993), suggesting a mechanism whereby attenuation of cAMP by L-AP4 may enhance NADPH oxidase activation in microglia.

Co-treatment of primary microglia with L-AP4 and thiorodazine or wortmannin increased superoxide production, suggesting that in primary microglia inhibition of Nox2 or Nox4 may increase superoxide production through activation of other Nox isoforms. Treatment of mesenchymal cells with L-AP4 mediates PI3-K signalling and NADPH oxidase activation (Wagner et al. 2007), and activation of the group III mGluRs is coupled to the p44/42ERK and PI3-K pathways in CGCs (Iacovelli et al. 2002), which induces Nox4 (Schröder et al. 2009) and Nox2 activation respectively (Ellson et al. 2006). These findings lend support to the data here, showing that activation of the microglial group III mGluRs induces Nox2 and Nox4 activation.

The $GABA_A$ receptor agonist muscimol significantly elevated Nox1 and Nox2 expression in primary microglia, which correlated with the functional data. Muscimol induced superoxide production was attenuated by co-treatment of BV2 microglia with apocynin, rottlerin or thioridazine, whist NADPH oxidase activity and superoxide production in primary microglia was significantly reduced by co-treatment with apocynin and rottlerin, therefore suggesting that $GABA_A$ receptor activation induces Nox1 and Nox2 activity and expression in a PKC dependent manner. As discussed previously, PKC activation modulates $GABA_A$ receptor activity (Moss et al. 1992), and activation of the $GABA_A$ receptor can induce PKC activation

(Brandon et al. 1999). Furthermore, in intact growth cones, $GABA_A$ receptor agonists induced PKC activation and phosphorylation of down-stream signalling molecules activated by a $GABA_A$ receptor mediated Ca^{2+} influx (Fukura et al. 1996). ROS also modulate $GABA_A$ receptor activity and its release of Cl^- ions from CA1 pyramidal neurons (Sah et al. 2002), suggesting a close interaction between ROS production through $GABA_A$ receptor activation, subsequent Cl^- release and Ca^{2+} influx, and PKC activation, which may promote and perpetuate superoxide production through the activation (Byun et al. 2008) and enhanced expression (Wei et al. 2010) of Nox1. The data presented here therefore suggest that activation of the microglial $GABA_A$ receptor favours Nox1 and Nox2 activation through PKC signalling.

Activation of the microglial P2Y2/4 receptor enhanced Nox2 and Nox4 expression. Furthermore, P2Y2/4 receptor induced superoxide production was attenuated by co-treatment of primary microglia with apocynin, suggesting Nox2 activation, whilst P2Y2/4 receptor induced superoxide production in BV2 microglia was attenuated by co-treatment with all inhibitors. Treatment of primary microglia with UTPγS and rottlerin significantly increased superoxide production, which could suggest that Nox1 inhibition may induce the activity of other Nox isoforms, and also lends support to the findings that in microglia, co-treatment with UTPγS and apocynin induced a significant increase in Nox4 expression, suggesting that inhibition of superoxide generated by Nox1/2 may promote enhanced Nox4 expression and activity (Byrne et al 2003).

In other cell types, P2Y2/4 receptor activation induces PI3-K activity (Kaczmarek et al. 2005), which activates Nox2 (Ellson et al. 2006), and was shown here to be implicated in P2Y2/4 receptor mediated superoxide production in BV2 microglia. Furthermore, in astrocytes P2Y2 receptor activation induces chemotaxis in a PI3-K and MAPK dependent manner (Wang et al. 2005). PI3-K did not however mediate UTPγS induced superoxide

production in primary microglia. In contrast, activation of microglial P2Y receptors induces PLC activation, through promoting calcium release from intracellular stores (Illes et al. 1996), and PLC mediates Nox2 activation in macrophages (Bae et al. 2009), thereby suggesting that in primary microglia Nox2 activation may be induced by PLC rather than PI3-K signalling after P2Y2/4 receptor activation as seen in BV2 microglia.

Preliminary investigations were conducted into NADPH oxidase isoform expression following iGluR modulation. AMPA receptor inhibition significantly increased Nox1 expression. No functional analyses were performed, however, it has been suggested in the literature that the modulation of the AMPA and NMDA receptors promotes ROS production through Nox4 activation in primary cortical neurons (Ha et al. 2010). The data presented here therefore suggest that NMDA and AMPA receptor activation may induce activation rather than expression of the NADPH oxidase.

These data suggest that modulation of neurotransmitter receptors affects the expression and activity of different NADPH oxidase isoforms, and that the activity of each NADPH oxidase isoform is modulated predominantly through activation of kinases following neurotransmitter receptor modulation, which promote the phosphorylation of cytoplasmic subunits of the NADPH oxidase.

4.3.2 The MAPK signalling pathway is implicated in neurotransmitter receptor induced NADPH oxidase activity

The Microglial MAPK signalling pathway is activated in several neurodegenerative conditions. Both p38MAPK and p44/42ERK are downstream of PKC and PI3-K, and are implicated in microglial reactivity, with ramifications for neuronal survival during neurodegeneration (Koistinaho & Koistinaho 2002). The involvement of the p38MAPK and

p44/42ERK signalling cascades in NADPH oxidase dependent superoxide production as a consequence of neurotransmitter receptor modulation were therefore investigated.

Inhibition of the microglial group I mGluR induced superoxide production and NADPH oxidase activity that was attenuated by p44/42ERK inhibition, however, NADPH oxidase activity was significantly increased after p38MAPK inhibition. Inhibition of mGluR5 with MTEP decreases the expression of signalling molecules in the MAPK pathway in the rat frontal cortex (Gass & Olive 2008). Furthermore, MTEP treatment down-regulates p44/42ERK phosphorylation in an *in vivo* rat model of PD (Rylander et al. 2009), suggesting that MTEP inhibits the MAPK signalling pathway. This correlates with findings that group I mGluR activation with DHPG increases p44/42ERK phosphorylation (Peavy & Conn 1998). The finding here that inhibition of the microglial group I mGluR induces superoxide production which can be attenuated by inhibition of p44/42ERK is therefore at odds with the published data, and could suggest that it is not direct inhibition of the group I mGluRs that induces superoxide production, but that group I mGluR inhibition may induce the release of factors that can feedback onto microglia and induce NADPH oxidase activation. Antagonism of the group I mGluRs with MTEP induces a reactive microglial phenotype (Byrnes et al. 2009), which promotes the release of neurotoxic factors such as TNFα and glutamate (Pocock &Kettenmann 2007), which feedback onto TNFRs (Mir et al. 2009) and other glutamate receptors (Harrigan et al. 2008) to induce NADPH oxidase activation. Furthermore, activation of microglial group I mGluRs following treatment with LPS attenuates TNFα release, which could be enhanced by mGluR group I antagonists (Farso et al. 2009), lending support to the suggestion that here, group I mGluR antagonism may induce TNFα release, which could feedback onto microglia and induce NADPH oxidase activation through p44/42ERK activation. In support of this, activation with CD40 ligand (a member of the TNF superfamily) promotes microglial reactivity and p44/42MAPK activation (Tan et al. 2000)

and microglial activation can induce p44/42ERK phosphorylation (Pocock & Liddle 2001), which in turn induces superoxide production through NADPH oxidase activation (Schröder et al. 2009). This could therefore suggest that MTEP mediates superoxide production in an indirect manner through induction of microglial reactivity and subsequent p44/42ERK activation.

To further support the role of p44/42ERK in MTEP induced NADPH oxidase activation, Western blot analysis showed that MTEP treatment increased p44 phosphorylation in an NADPH oxidase dependent manner. ROS mediates p44/42ERK activity (Anilkumar et al. 2008); therefore antagonism of the group I mGluR may induce NADPH oxidase activation through p44/42ERK activation, (Fig. 4.15), which could have important consequences for sustained superoxide production in microglia.

Microglial mGluR3 - induced superoxide production was attenuated by p38MAPK and p44/42ERK inhibition. Activation of the astrocytic mGluR3 mediates p44/42ERK activation, which is protective after ischaemia (Ciccarelli et al. 2007), whilst mGluR3 inhibition in gliomas attenuates p44/42ERK activation and glioma growth *in vitro* (D'Onofrio et al. 2003). Activation of mGluR3 therefore induces p44/42ERK activation, which was shown here to be implicated in NADPH oxidase mediated superoxide production. In addition, Western blot analysis revealed that mGluR3 activation promoted p44ERK and p38MAPK phosphorylation in an NADPH oxidase dependent manner (Fig. 4.15). Furthermore, activation of the astrocytic mGluR3 induces p44/42ERK and p38MAPK phosphorylation, which has neuroprotective consequences (D'Onofrio et al. 2001).

The suggestion that NADPH oxidase induced ROS can stimulate MAPK activation is supported by findings in astrocytes in which NADPH oxidase activity following treatment with the known NADPH oxidase activator menadione stimulates p44/42ERK and p38MAPK

phosphorylation, which could be attenuated by apocynin (Zhu et al. 2009). These reports therefore lend support to the suggestion that here, mGluR3 activation promotes NADPH oxidase activation and ROS production through p44/42ERK and p38MAPK phosphorylation, and that this mGluR3 mediated ROS production can also modulate the activity of MAPK signalling molecules.

Group III mGluR induced superoxide production was attenuated by p38MAPK inhibition; however, Western blot analysis showed that p38MAPK phosphorylation was not up-regulated by L-AP4 treatment, although activation of the group III mGluRs induced p44ERK phosphorylation in an NADPH oxidase dependent manner. Activation neuronal group III mGluRs promotes p44/42ERK activation (Iacovelli et al. 2002), and in microglia, activation of group III mGluRs attenuates microglial reactivity (Taylor et al. 2003). Microglial group III mGluR activation may therefore induce superoxide production through p38MAPK activation, which mediates p44ERK phosphorylation, which could have neuroprotective consequences. This suggestion is supported by studies in astrocytes, in which ERK activation promotes the protection of neurons through the release of glial derived neurotrophic factor (GDNF) (Chu et al. 2008), which should be investigated further in microglia treated with L-AP4.

Microglial $GABA_A$ receptor - induced superoxide production and NADPH oxidase activity was inhibited by p38MAPK and p44/42ERK inhibition (Fig. 4.15). However, muscimol induced ROS had no effect on p38MAPK or p44/42ERK phosphorylation, suggesting that $GABA_A$ receptor induced superoxide production could not perpetuate MAPK signalling. $GABA_A$ receptor activation is modulated by p38MAPK and p44/42ERK activity (Bell-Horner et al. 2006), which suggests that MAPK signalling exacerbates GABA receptor induced superoxide production. The findings here that activation of the $GABA_A$ receptor induced Nox2 expression and activity could imply that there is cross-talk between Nox2 derived ROS and the p44/42ERK signalling pathway (Song et al. 2011), as inhibition of this

signalling molecule reduced superoxide production, suggesting that activation of the $GABA_A$ receptor may mediate superoxide production in a p44/42ERK dependent manner. Muscimol also promotes Nox4 activation and superoxide production through p44/42ERK signalling (Tyagi et al. 2009). Furthermore, Nox4 is regulated by p44/42ERK in preadipocytes (Schröder et al. 2009), and Nox4 mediated ROS can also modulate p44/42ERK phosphorylation in vascular cells (Gorin et al. 2004; Anilkumar et al. 2008). The data presented here therefore suggest that $GABA_A$ receptor activation promotes MAPK signalling, which induces NADPH oxidase mediated superoxide production.

P2Y2/4 receptor induced superoxide production was attenuated by p38MAPK inhibition, whilst p44/42ERK inhibition increased NADPH oxidase activity. P2Y2/4 receptor activation in chromaffin cells mediates p44/42ERK phosphorylation (Luke & Hexum 2008), which corresponds with the Western blot analysis presented here. Furthermore, stimulation of the neuronal P2Y receptor promotes p44/42ERK phsophorylation, which mediates neurite outgrowth (Lakshmi &Joshi 2006) and Nox4 activation (Schröder et al. 2009). The data presented here therefore suggests that P2Y2/4 receptor activation may induce superoxide dependent phosphorylation of p44/42ERK, however p44/42ERK may not regulate NADPH oxidase activation in microglia, as inhibition of p44/42ERK could not attenuate superoxide production. Instead, P2Y2/4 induced p44/42ERK activation, mediated by NADPH oxidase activity, may promote microglial reactivity, which could further enhance superoxide production through the release of cytokines which feedback onto microglia to exacerbate activation (Mir et al. 2009).

In mesengial cells, P2Y2/4 receptor activation with UTP mediates p38MAPK activation (Huwiler et al. 2000), therefore supporting the findings here that p38MAPK inhibition attenuated P2Y2/4 receptor induced superoxide production (Fig. 4.15). The activation of p38MAPK is also associated with Nox2 induction through p67phox phosphosylation (Dang

et al. 2003), which corresponds with findings here that P2Y2/4 receptor activation induces Nox2 activity.

Modulation of the iGluRs induced superoxide production in a p38MAPK and p44/42ERK dependent manner. Superoxide production in primary microglia following NMDA treatment was attenuated by p38MAPK and p44/42ERK inhibition. Furthermore, p38MAPK phosphorylation was enhanced by NMDA induced superoxide production in an NADPH oxidase dependent manner, suggesting that NMDA induced superoxide production modulates p38MAPK activation (Fig. 4.15). In VSMC's, NMDA receptor activation promotes p38MAPK activation (Doronzo et al. 2010). Furthermore, NMDA induced ROS production promotes p38MAPK phosphorylation in cortical neurons (Park et al. 2004). NMDA therefore induces superoxide production through p38MAPK activation, and NMDA receptor induced ROS also modulates p38MAPK activation, in support of the data presented here. AMPA receptor activation induced superoxide production in a p38MAPK dependent manner; however inhibition of the AMPA receptor also increased p38MAPK phosphorylation. Activation of p38MAPK following AMPA receptor activation is seen after injury in astrocytes (Zhou et al. 2011). It could therefore be suggested that AMPA receptor activation may occur following injury and excess glutamate levels, which promotes p38MAPK activation and superoxide production.

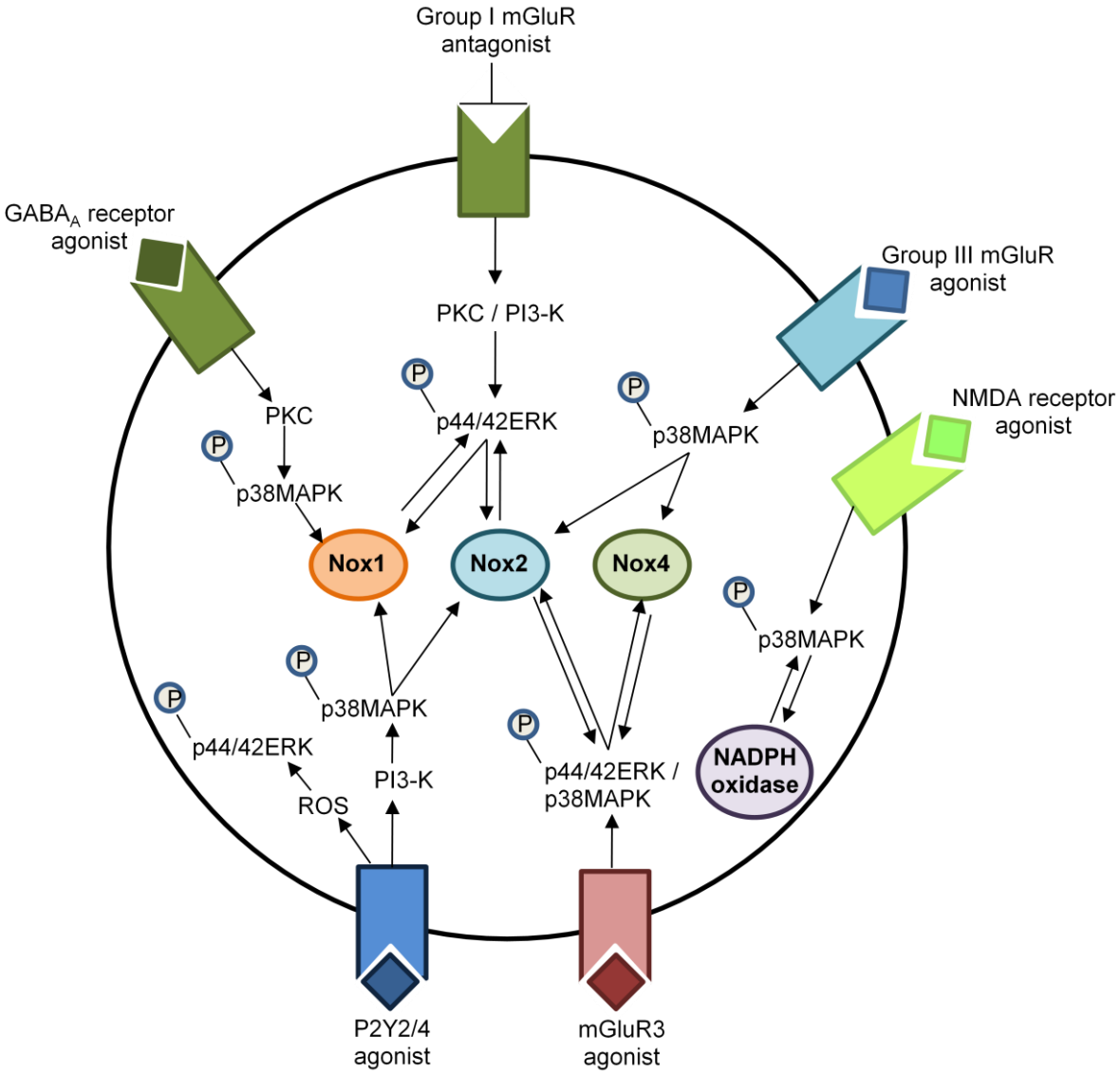

Figure 4.15 Schematic of mechanisms of neurotransmitter mediated Nox isoform activation. *The data in this chapter show that inhibition of the group I mGluR with MTEP induces PKC and PI3-K activation. Inhibition of p44/42ERK attenuated superoxide production, and superoxide production was shown to induce an increase in p44ERK phosphorylation. This pointed to the activation of Nox1 and Nox2 by inhibition of group I mGluRs. Activation of the group III mGluR with L-AP4 induced an increase in superoxide production that could be attenuated by inhibition of PI3-K and PKC. Inhibition of p38MAPK also attenuated superoxide production, which suggested that L-AP4 induced the activity of Nox2 and Nox4. Superoxide production induced by NMDA receptor activation could be attenuated by inhibition of p38MAPK, and activation of NMDA receptors induced phosphorylation of p38MAPK. It was not known which isoform of the NADPH oxidase could be induced by NMDA receptor activation. Activation of mGluR3 with NAAG induced superoxide production that could be attenuated by inhibition of p44/42ERK or p38MAPK. NAAG induced superoxide production also increased p44ERK and p38MAPK phosphorylation, and pointed to the involvement of Nox2 and Nox4. Activation of the P2Y2/4 receptor with UTPγS induced superoxide production that could be attenuated by inhibition of PI3-K and could also be attenuated by inhibition of p38MAPK , suggesting activation of Nox1 and Nox2. P2Y2/4 receptor induced superoxide production also enhanced the phosphorylation of p44/42ERK. Activation of the GABA$_A$ receptor induced the activity of PKC and superoxide production could also be attenuated by inhibition of p38MAPK, suggesting that GABA$_A$ receptor activation induces the activity of Nox1.*

4.3.3 Neurotransmitter induced NADPH oxidase activity and microglial reactivity

The findings that modulation of neurotransmitter receptors affected p44/42ERK and p38MAPK activity and NADPH oxidase activation, lead to an investigation into microglial reactivity. Activation of the p38MAPK and p44/42ERK signalling pathways are implicated in an activated microglial phenotype (Pocock and Liddle 2001). Microglia exhibit an activated phenotype in AD, which is associated with enhanced p38MAPK activation in a transgenic mouse model of the disease (Giovannini et al. 2002). Furthermore, activation of microglia with LPS elevates p38MAPK activity and promotes TNFα release (Nakajima et al. 2004). In addition microglial p44/42ERK activation by paraquat induces a neurotoxic phenotype following upregulation of the NADPH oxidase (Miller et al. 2007), and traumatic spinal cord injury mediates microglial reactivity and a neurotoxic phenotype associated with p44/42ERK activation (Xu et al. 2006). The findings here that modulation of microglial neurotransmitter receptors could induce activation of the p38MAPK and p44/42ERK signalling pathways therefore prompted an investigation into microglial reactivity.

Glutamate and BzATP increased microglial ED1 expression, which is a marker for phagocytosis, however, iNOS expression was not induced by neurotransmitter treatment. Phagocytosis is upregulated following exposure of microglia to ATP and UTP (Inoue 2007) , however short term exposure to ATP (30 min) attenuates phagocytoisis (Fang et al. 2009). Here however, treatment was for 24 h, and therefore long enough to induce ED1 up-regulation. Treatment with glutamate also increased this phagocytic phenotype. Activation of microglia with kainate promotes cytoskeletal rearrangements seen during phagocytosis, suggesting that microglial AMPA receptor activation mediates a phagocytic phenotype (Christensen et al. 2006), and that glutamate treatment therefore promotes a phagocytic phenptype.

Treatment of microglia with the mGluR modulators MTEP, NAAG and L-AP4 significantly elevated iNOS expression, which is a marker of microglial activation. Inhibition of group I mGluRs promotes a reactive microglial phenotype (Byrnes et al. 2009), however it is not yet known whether p44/42ERK activation is implicated. Activation of mGluR3 is also associated with microglial reactivity (Taylor et al. 2002), however activation of group III mGluRs promotes neuroprotection (Taylor et al. 2003), whilst here, an increase in iNOS expression was observed. However, as all three mGluR modulators induce p44/42ERK up-regulation, this could induce a reactive phenotype. Activation of the microglial P2Y2/4 receptor also enhanced iNOS expression, which correlates with suggestions that UTP can mediate microglial chemotaxis and phagocytosis (Inoue et al. 2007), which is suggestive of a reactive microglial phenotype.

4.3.4 Conclusions

The data presented in this chapter show that modulation of microglial neurotransmitter receptors induces the activity of different NADPH oxidase isoforms, and that modulation of neurotransmitter receptors and subsequent NADPH oxidase isoform activity requires and can induce p44/42ERK and p38MAPK activity, which modulates microglial reactivity. The ramifications of neurotransmitter and receptor induced Nox isoform activity and subsequent microglial activation on neuronal survival have been investigated further in the next chapter.

Chapter 5

Ramifications of neurotransmitter and receptor mediated microglial NADPH oxidase activity on neuronal survival

5.1 Introduction and summary of results

In the previous chapter, modulation of microglial neurotransmitter receptors was shown to induce the activity of different NADPH oxidase isoforms, and could also modulate MAPK signalling and microglial reactivity. As the activation of different Nox isoforms can induce the release of neurotoxic factors (Chéret et al. 2008), and as activation of the MAPK signalling pathway mediates microglial reactivity (Pocock and Liddle, 2001), it was important to investigate the ramifications of microglial neurotransmitter receptor modulation and NADPH oxidase activation on neuronal survival, which may have important consequences for future therapies.

The production and release of the cytokine TNFα from microglia treated with neurotransmitters and receptor modulators was investigated, as this cytokine has important ramifications for neuronal survival and is regulated by NADPH oxidase activation. TNFα is implicated in immune surveillance, homeostasis and defence against damaging insults in the CNS (Sriram & O'Callaghan 2007), as well as promoting neuronal recovery after traumatic injury (Scherbel et al. 1999). However, TNFα is also a pro-inflammatory cytokine, released from activated microglia following CNS damage in neurodegenerative diseases, infection, and neurotoxicity (Mattson et al. 1997), resulting in neuronal damage (Sriram & O'Callaghan 2007).

Microglia produce TNFα following activation with a variety of stimuli (Mattson et al. 1997), and modulation of the microglial NADPH oxidase mediates TNFα release, which can feedback onto microglial TNFR's to perpetuate NADPH oxidase mediated superoxide production, through Nox1 activation (Kim et al. 2007). Furthermore, microglial NADPH oxidase activation and intracellular ROS production mediates TNFα expression in a MAPK and PKC dependent manner (Qin et al. 2004). Nox2 activation in spinal cord microglia after nerve injury induces ROS production which mediates TNFα release, and contributes to

neuropathic pain (Kim et al. 2010). Furthermore, Aβ induced microglial NADPH oxidase activation promotes TNFα production in a ROS dependent manner, which contributes to neurodegeneration (Block 2008), suggesting the importantce of NADPH oxidase mediated TNFα production in disease.

TNFα release from microglia can be regulated by neurotransmitter receptor modulation, with ramifications for neuronal survival (Pocock and Kettenmann, 2007) (Fig. 5.1). Activation of the microglial group I mGluRs can reduce TNFα production and release induced by LPS, which down-regulates microglial reactivity, and is neuroprotective (Farso et al. 2009) (Fig. 5.1). In contrast, stimulation of mGluR2, promotes TNFα and Fas ligand release (Fig. 5.1), which induces neuronal apoptosis, however activation of the microglial mGluR3 with NAAG does not mediate TNFα release or neuronal death (Taylor et al. 2005). Microglial group III mGluR activation is neuroprotective through attenuation of the release of stable neurotrophic factors (Fig. 5.1), and L-AP4 treatment of microglia reduces LPS, CgA, or $A\beta_{1-42}$ induced microglial activation (Taylor et al. 2003). Activation of microglial NMDA receptors promotes TNFα release with neurotoxic consequences (Zhu et al. 2010) (Fig. 5.1), and inhibition of the microglial NMDA receptor with MK-801 down-regulates TNFα release in a mouse model of hypoxia (Murugan et al. 2011). Activation of the AMPA receptor in primary microglia mediates TNFα production, however the ramifications for neuronal survival are not yet known (Noda et al. 2000).

There are few reports regarding the effects of GABA receptor modulation on TNFα production and neuronal survival, however, antagonism of the microglial $GABA_A$ receptor with DHEAS (an allosteric antagonist of the $GABA_A$ receptor) can reduce TNFα release (Di Santo et al. 1996) (Fi. 5.1).Microglial P2X7 receptor activation with BzATP promotes TNFα release which is protective against neuronal excitotoxic injury (Suzuki et al. 2004) through its binding to neuronal TNFR2 which mediates p38MAPK signalling, shown to favour neuronal survival (Yang et al. 2002). Activation of microglial P2Y receptors also promotes microglial TNFα release, which may be protective (Ogata et al. 2003) (Fig. 5.1).

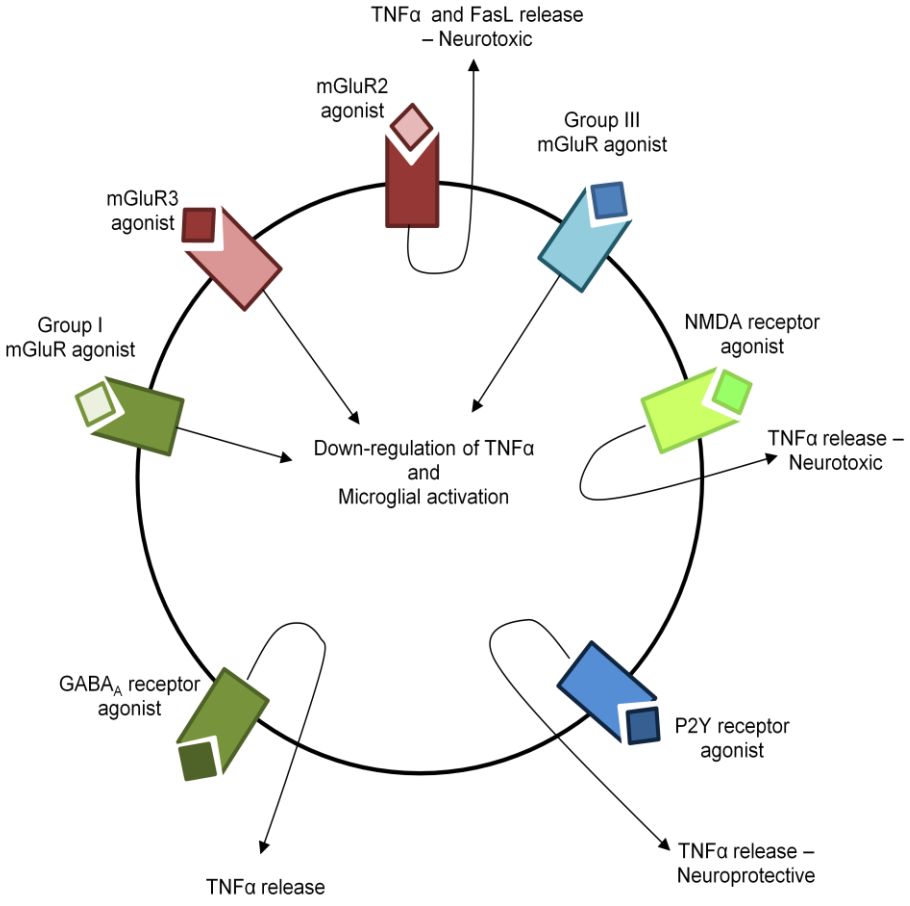

Figure 5.1 Schematic of known ramifications of microglial neurotransmitter receptor modulation. *Activation of the group I mGluRs, the group III mGluRs and mGluR3 are protective through the down-regulation of microglial activation and TNFα production. Activation of the NMDA receptor and mGluR2 mediates TNFα release and neuronal apoptosis. Activation of the microglial P2Y receptors mediates TNFα release with protective consequences; whilst the ramifications of $GABA_A$ receptor mediated release of TNFα is as yet unknown.*

As modulation of microglial neurotransmitter receptors can regulate TNFα release and neuronal survival, it was important to investigate whether this was dependent on NADPH oxidase activation. Initially, microglial TNFα production and release was investigated. Treatment of microglia with glutamate, GABA or BzATP induced TNFα expression but not release. Activation of the $GABA_A$ receptor with muscimol or the P2Y2/4 receptor with UTPγS significantly increased TNFα release in an NADPH oxidase dependent manner, and inhibition of superoxide production from microglia treated with the group I mGluR antagonist or the group III mGluR agonist significantly elevated TNFα gene expression, whilst protein expression was increased by treatment with the mGluR3 agonist NAAG and the group I antagonist MTEP in an NADPH oxidase dependent manner. Activation of the microglial AMPA receptor with QA significantly elevated TNFα expression in an NADPH oxidase dependent manner.

Induction of microglial TNFα release with muscimol, or TNFα expression with L-AP4 plus apocynin increased neuronal death in MGCM studies, which was mediated by neuronal caspase 12 cleavage and TNFR1 expression. Furthermore, microglial TNFα production after MTEP plus apocynin treatment decreased neuronal death, suggesting that this TNFα production is neuroprotective. Inhibition of mGluR3 induced superoxide production also attenuated neuronal death by decreasing caspase 12 cleavage, whilst AMPA receptor induced microglial superoxide production elevated neuronal apoptosis in MGCM studies in an NADPH oxidase dependent manner, suggesting a role for AMPA induced NADPH oxidase dependent TNFα production in neuronal apoptosis.

These data demonstrate that activation of the microglial NADPH oxidase through modulation of microglial neurotransmitter receptors affects neuronal survival through the release of neurotoxic or neurotrophic factors, which may be therapeutically relevant.

5.2 Results

5.2.1 Microglia express and release TNFα in an NADPH oxidase dependent manner following treatment with neurotransmitters and receptor agonists or antagonists

Intracellular superoxide production acts as a signalling molecule to promote the expression and release of cytokines from microglia (Qin et al. 2004; Turchan-Cholewo et al. 2009). As the release of soluble factors from microglia can contribute to neurotoxicity or protection (Li et al. 2009), it was considered important to investigate whether exposure of microglia to neurotransmitters or modulation of neurotransmitter receptors known to induce superoxide production, mediated cytokine release. Microglia express TNFα following activation of the NADPH oxidase (Jekabsone et al. 2006;Qin et al. 2004), therefore the expression and release of TNFα was investigated following treatment of microglia with neurotransmitters, and then following treatment with receptor agonists and antagonists.

Initially, microglia were treated with the neurotransmitters glutamate (1 μM), GABA (100 μM) or BzATP (250 μM), and the positive controls LPS (1 μg/ml), PMA (10 ng/ml) or the NADPH oxidase inhibitor apocynin (10 μM) for 24 h. TNFα expression was analysed by RT-PCR (Fig. 5.2Ai), and was normalised to β-actin to enable densitometry to be performed (Fig. 5.2Aii). Microglial TNFα release was investigated by ELISA (Fig. 5.2B) after treatment with the positive control LPS (1 μg/ml), and the neurotransmitters glutamate (1 μM), GABA (100 μM) or BzATP (250 μM) in the presence or absence of apocynin (10 μM) for 24 h.

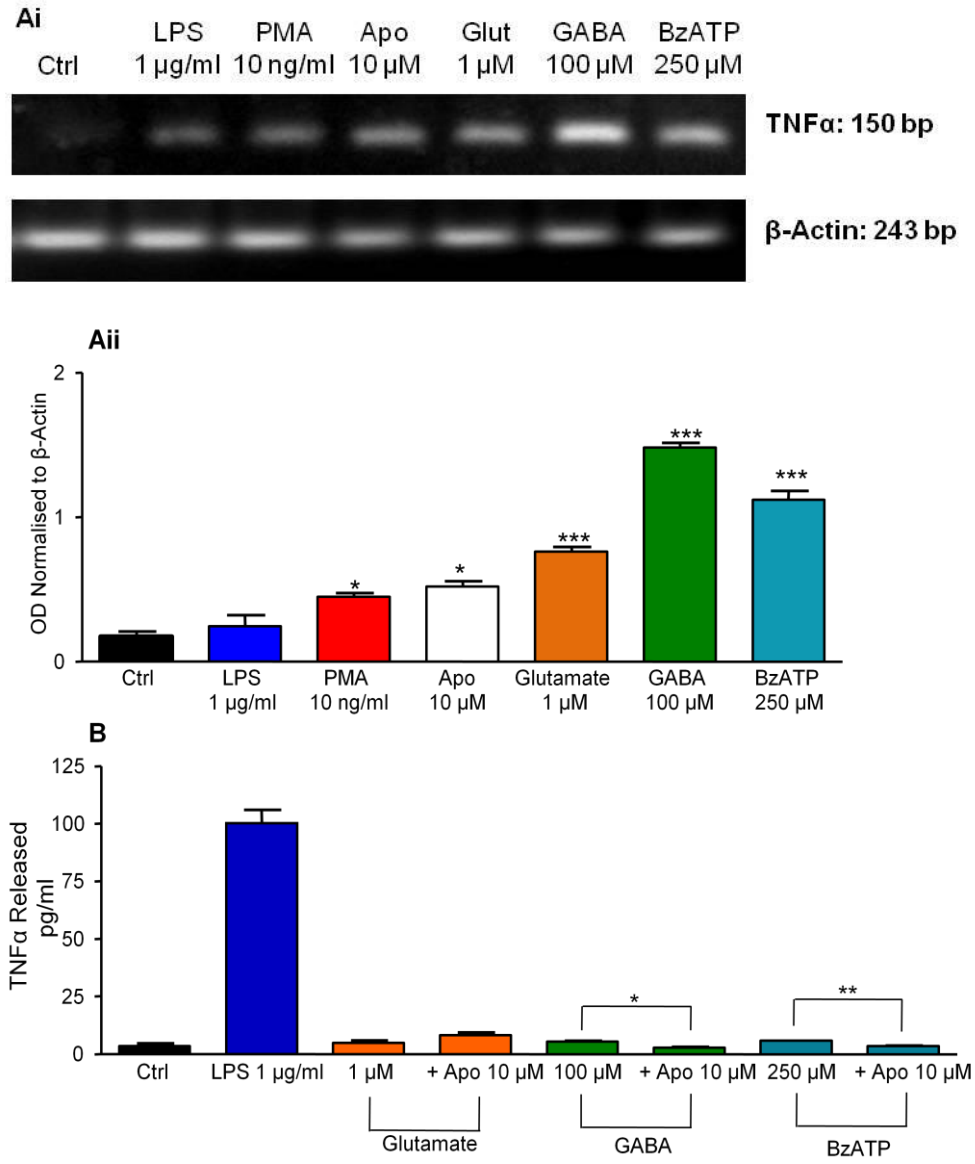

Figure 5.2 Production and release of TNFα by microglia treated with neurotransmitters. *The expression of TNFα was analysed in primary microglia by RT-PCR* ***(Ai, Aii).*** *Primary microglia were treated with the positive controls LPS (1 µg/ml), PMA (10 ng/ml), or apocynin (10 µM), or the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) for 24 h. Microglia were lysed and lysates were reverse transcribed to cDNA, which was subjected to PCR using primers for TNFα or β-actin* ***(Ai).*** *The RT-PCR was performed three times, using microglia from three separate preparations, enabling densitometry following normalisation to β-actin* ***(Aii).*** *TNFα release was analysed by ELISA following treatment of primary microglia with the positive control LPS (1 µg/ml), or the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence or absence of apocynin (10 µM) for 24 h* ***(B).*** *All experiments were performed in triplicate, and data were analysed by one way ANOVA and Tukey post-hoc analysis, comparing all treatments to control groups, and between treatments as shown. *p<0.05, **p<0.01, ***p<0.001.*

Glutamate (1 μM), GABA (100 μM) or BzATP (250 μM) each significantly elevated microglial TNFα expression when compared with control untreated cells (***$p<0.001$) (Fig. 5.2Ai, ii). PMA (10 ng/ml) or apocynin (10 μM) treatments also induced a significant increase in TNFα expression when compared with control untreated cells, however, this was less significant (*$p<0.05$) than treatment with neurotransmitters (***$p<0.001$). LPS (1 μg/ml) significantly elevated microglial TNFα release when compared with control untreated cells. Treatment with the neurotransmitters alone did not significantly increase TNFα release from microglia after 24 h treatment when compared with control untreated cells (Fig. 5.2B). GABA or BzATP induced TNFα release could be significantly reduced by co-treatment of microglia with apocynin (Fig. 5.2B), suggesting that any TNFα production following GABA or BzATP treatment may be dependent on NADPH oxidase activity.

TNFα gene and protein expression were next analysed in primary microglia treated with the neurotransmitter receptor agonists or antagonists. Primary microglia were treated with LPS (1 μg/ml), PMA (10 ng/ml) or apocynin (10 μM) or the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 μM), the group III agonist L-AP4 (100 μM), the $GABA_A$ receptor agonist muscimol (50 μM) or the P2Y2/4 receptor agonist UTPγS (100 μM) in the presence or absence of apocynin for 24 h. Microglia were lysed and analysed for TNFα gene expression by RT-PCR (Fig. 5.3Ai) and expression was normalised to β-actin for densitometry analysis (Fig. 5.3Aii). Protein expression was analysed by Western blotting (Fig. 5.3Bi), and expression was normalised to β-actin to enable densitometry to be performed (Fig. 5.3Bii).

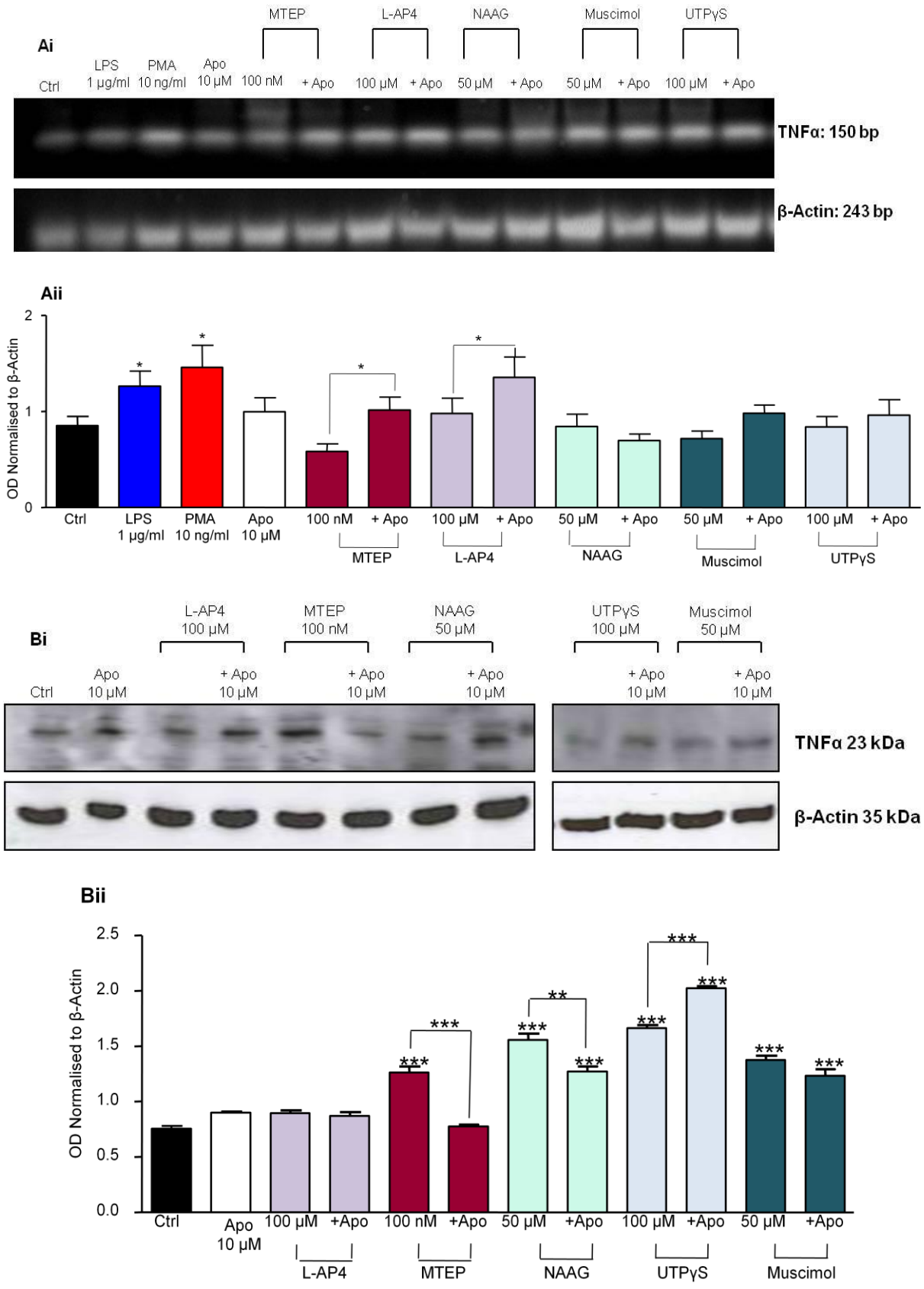

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Figure 5.3 TNFα expression in primary microglia treated with neurotransmitter receptor agonists or antagonists. *Primary microglia were treated with LPS (1 µg/ml), PMA (10 ng/ml), apocynin (10 µM) or the mGluR group I antagonist MTEP (100 µM), the group III agonist L-AP4 (100 µM), the mGluR3 agonist NAAG (50 µM), the $GABA_A$ receptor agonist muscimol (50 µM) or the P2Y2/4 receptor agonist UTPγS (100 µM) in the presence or absence of apocynin for 24 h. Microglia were then lysed and reverse transcribed to cDNA before PCR using TNFα primers and also primers for the loading control β-actin* ***(Ai).*** *Densitometry was performed to semi-quantify TNFα expression levels after normalisation to β-actin* ***(Aii).*** *Western blot analysis was also performed to investigate TNFα protein levels following treatment of microglia with apocynin (10 µM), or L-AP4 (100 µM), MTEP (100 nM), NAAG (50 µM), UTPγS (100 µM) or muscimol (50 µM) in the presence or absence of apocynin for 24 h* ***(Bi).*** *Lysates were run on a 10% SDS-PAGE, and were transferred to a PVDF membrane. Following blocking, the membrane was incubated with the goat-anti-rabbit TNFα primary antibody (1:1000) at 4 °C overnight, before incubation with the secondary antibody, goat-anti-rabbit-HRP (1:2000) for 1 h at room temperature. The membrane was visualised by ECL. The membrane was then stripped and re-probed with the rabbit-anti-mouse β-actin antibody (1:2000) for 2 h at room temperature, before incubation with the rabbit-anti-mouse HRP secondary antibody (1:1000) for 1 h at room temperature. The membrane was visualised by ECL. This enabled densitometry to be performed* ***(Bii).*** *Densitometry data were analysed by one way ANOVA and Tukey post-hoc analysis in which all treatments were compared with control untreated cells, or comparisons were made between cells treated with agonists / antagonists alone or in the presence of apocynin. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

Treatment of microglia with PMA or LPS significantly increased TNFα gene expression when compared with control untreated cells (Fig. 5.3Ai, ii). Treatment with the neurotransmitter agonists or antagonists alone did not elevate TNFα expression (Fig. 5.3Ai, ii), however, co-treatment of microglia with MTEP and apocynin or L-AP4 and apocynin significantly increased TNFα expression when compared with MTEP or L-AP4 treatment alone respectively (Fig. 5.3Ai, ii), suggesting that inhibition of superoxide production mediates TNFα transcription.

Treatment of primary microglia with the group I mGluR antagonist MTEP or the mGluR3 agonist NAAG significantly increased TNFα protein levels when compared with control untreated cells, in an NADPH oxidase dependent manner (Fig. 5.3Bi, ii). Treatment with the P2Y2/4 receptor agonist UTPγS or the $GABA_A$ receptor agonist muscimol significantly elevated TNFα protein expression, which could be further enhanced by co-treatment with apocynin, suggesting that inhibition of P2Y2/4 or $GABA_A$ receptor induced superoxide production enhances TNFα expression (Fig. 5.3Bi, ii).

TNFα release following treatment of microglia with the receptor agonists or antagonists was next investigated (Fig. 5.4). Primary microglia were treated with the positive control LPS (1 μg/ml) or the group III mGluR agonist L-AP4 (100 μM), the group I mGluR antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 μM), the $GABA_A$ receptor agonist muscimol (50 μM) or the P2Y2/4 receptor agonist UTPγS (100 μM) in the presence or absence of apocynin for 24 h before the culture media was removed and used to analyse TNFα release by ELISA (Fig. 5.4).

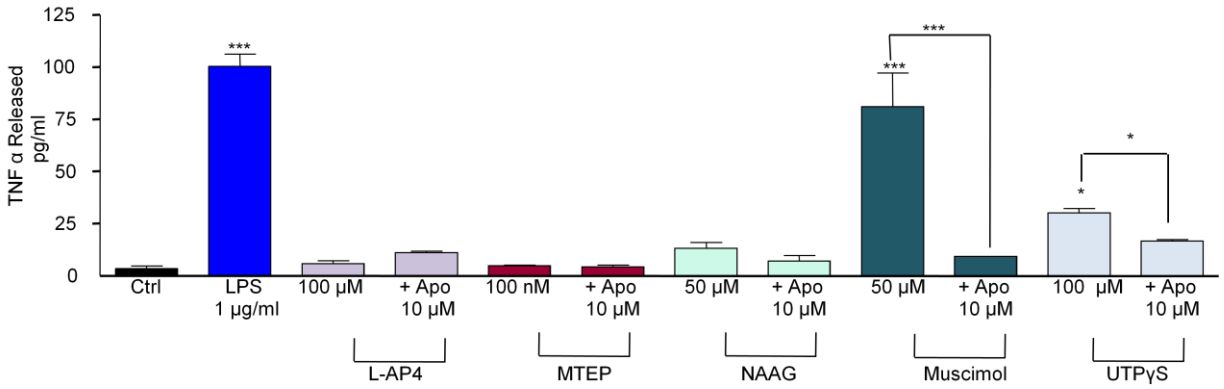

Figure 5.4 TNFα release from microglia treated with neurotransmitter receptor agonists or antagonists. *Primary microglia were treated with LPS (100 µM), or the mGluR group III agonist L-AP4 (100 µM), the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 µM), the $GABA_A$ receptor agonist muscimol (50 µM), or the P2Y2/4 receptor agonist UTPγS (100 µM) in the presence or absence of apocynin (10 µM) for 24 h. Media was then removed from the cells and assessed for TNFα release using a TNFα ELISA. Data were analysed using a one way ANOVA with Tukey post-hoc analysis, and comparisons were made between treatments and control untreated microglia, and also between cells treated alone and in the presence of apocynin. *p<0.05, **p<0.01, ***p<0.001. Data are n=3.*

TNFα release was significantly elevated by treatment of primary microglia with LPS (1 μg/ml) (Fig. 5.4). Treatment of primary microglia with the mGluR group III agonist L-AP4, the mGluR group I antagonist MTEP, or the mGluR3 agonist NAAG alone or in the presence of apocynin did not modulate TNFα release, however activation of the $GABA_A$ receptor with muscimol or the P2Y2/4 receptor with UTPγS significantly increased TNFα release when compared with control untreated cells, which could be attenuated by co-treatment of microglia with apocynin, suggesting that muscimol and UTPγS induced superoxide production mediates TNFα release (Fig. 5.4).

TNFα expression in primary microglia treated with iGluR modulators was next investigated. Primary microglia were treated with the NMDA receptor agonist NMDA (100 μM) or the antagonist MK-801 (10 μM) either alone, in combination, or with apocynin (10 μM); or the AMPA receptor agonist QA (10 μM) or the antagonist CNQX (10 μM) either alone, in combination, or in the presence of apocynin for 24 h, before RT-PCR was performed using primers for TNFα and β-actin (Fig. 5.5A) to enable densitometry to be performed (Fig. 5.5B).

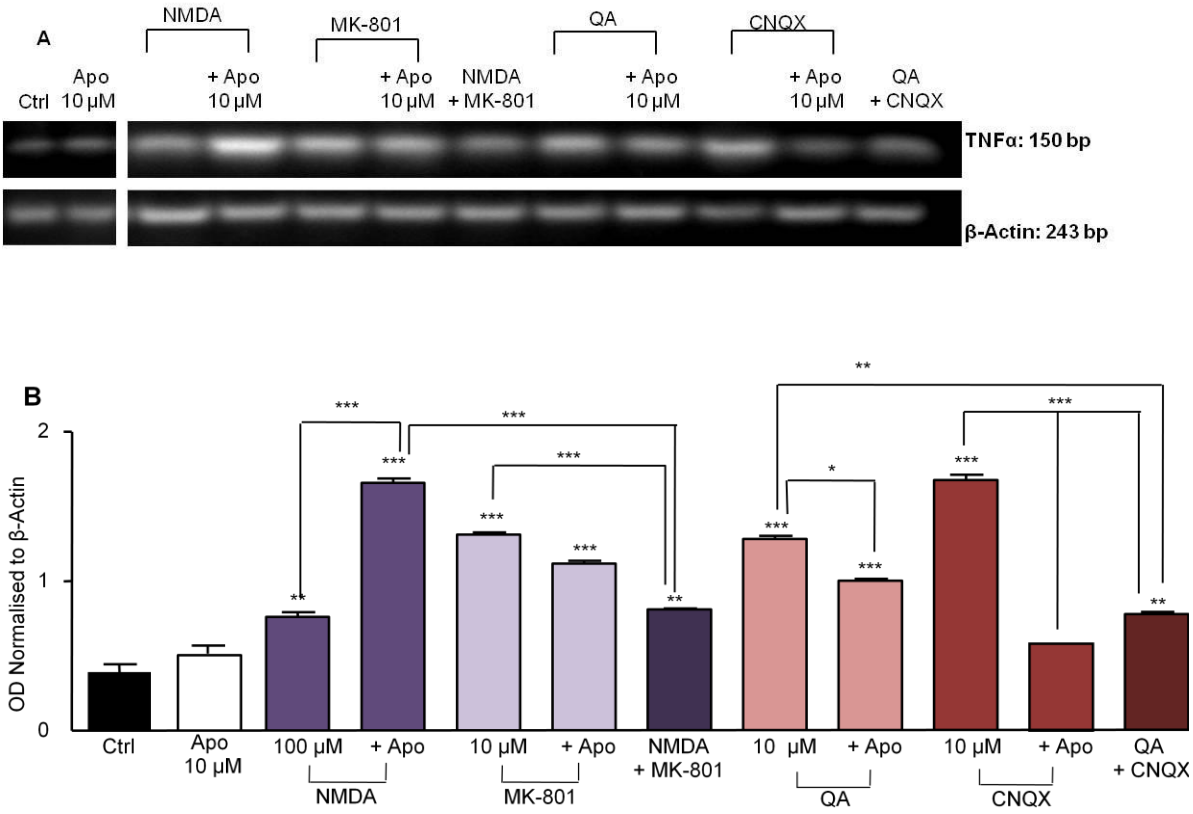

Figure 5.5 TNFα expression in primary microglia after modulation of iGluRs. *Primary microglia were treated with the NMDA receptor agonist NMDA (100 µM), or the antagonist MK-801 (10 µM), either alone, in combination or in the presence of apocynin (10 µM); or were treated with the AMPA receptor agonist QA (10 µM) or the antagonist CNQX (10 µM) either alone, in combination, or in the presence of apocynin (10 µM) for 24 h. Microglia were also treated with apocynin alone. Microglia were then lysed and subjected to RT-PCR using primers for TNFα or β-actin* ***(A).*** *Densitometry was performed to determine expression levels of TNFα normalised to β-actin* ***(B).*** *Data were analysed by one way ANOVA and Tukey post-hoc analysis, making comparisons between treatments and control untreated cells, and between treatments as shown. *p<0.05, **p<0.01, ***p<0.001. All data are n=3.*

NMDA significantly enhanced TNFα expression when compared with control untreated cells, which could be further increased upon co-treatment with apocynin (Fig. 5.5A, B), suggesting that inhibition of NMDA receptor induced superoxide production enhances TNFα expression. Inhibition of the NMDA receptor with MK-801 also enhanced TNFα expression although in an NADPH oxidase independent manner, however TNFα expression could be significantly attenuated upon co-treatment of microglia with NMDA and MK-801 (Fig. 5.5B). Activation of the AMPA receptor significantly enhanced TNFα expression when compared with control untreated cells which could be significantly attenuated by co-treatment with apocynin. Furthermore, this AMPA receptor induced increase in TNFα expression could also be significantly attenuated by co-treatment with the receptor agonist and antagonist (Fig. 5.5B). The AMPA receptor antagonist CNQX also enhanced microglial TNFα expression, which could be attenuated by co-treatment with apocynin or upon co-treatment with QA, suggesting that inhibition of the AMPA receptor enhances TNFα expression in an NADPH oxidase dependent manner.

These data show that neurotransmitter receptor agonists or antagonists can modulate TNFα expression through activation of the NADPH oxidase. These findings therefore prompted an investigation into the ramifications of microglial neurotransmitter receptor induced NADPH oxidase activation on neuronal survival.

5.2.2 Microglial conditioned media (MGCM) from microglia treated with neurotransmitters and receptor agonists or antagonists modulates neuronal survival in an NADPH oxidase dependent manner

Following the findings that modulation of microglial neurotransmitter receptors affected TNFα expression in an NADPH oxidase dependent manner, it was important to determine whether the release of toxic or protective factors from microglia treated in this way affected neuronal survival. The effects of MGCM from microglia treated with neurotransmitters or receptor modulators in the presence or absence of apocynin were first investigated, before an analysis of survival and death cascades in neurons treated with MGCM were conducted.

To investigate the effects of neurotransmitter mediated microglial superoxide production on neuronal survival, microglia were treated with the neurotransmitters glutamate (1 μM), GABA (100 μM) or BzATP (250 μM) in the presence or absence of apocynin for 24 h (Fig. 5.6A). CGCs grown for 7 DIV were then incubated with MGCM for 24 h and death was assessed by fluorescence microscopy using PI, a marker of necrosis and late apoptosis, and the nuclear dye Hoechst 33342, for the detection of all cells in the field (Fig. 5.6 Aii). Cells were counted and were expressed as a percentage of dead (PI positive) cells (Fig. 5.6Ai). To investigate the role of Nox4 in neurotransmitter induced neuronal apoptosis, microglia were treated with the neurotransmitters as above, in the presence or absence of the Nox4 inhibitor thioridazine (1 μM) for 24 h before media was applied to CGCs for a further 24 h, and cell death was assessed as described (Fig. 5.6B). To ensure that excess neurotransmitters in the MGCM were not responsible for the effects observed, and therefore to be certain that the consequences for neuronal survival were due to neurotransmitter induced release of neurotoxic or neurotrophic factors from microglia, CGCs were treated directly with the neurotransmitters in the presence or absence of apocynin as described for 24 h, and were assessed for death using PI (Fig. 5.6C).

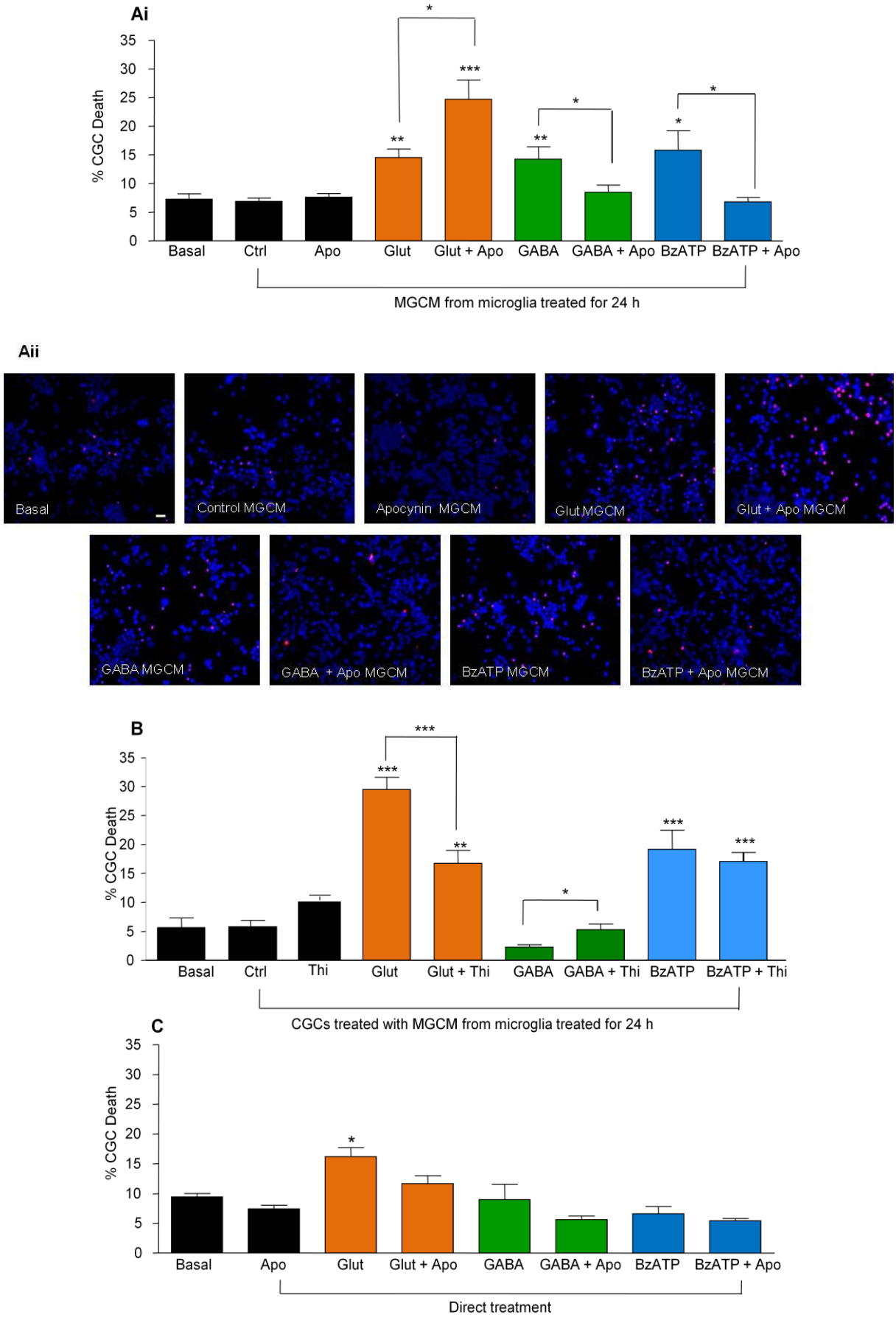

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Figure 5.6 CGC death after treatment with MGCM from microglia treated with neurotransmitters in the presence or absence of apocynin. *Microglia were treated with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence or absence of apocynin (10 µM) for 24 h. Culture medium was removed and placed onto CGC's grown for 7 DIV, and neurons were incubated for 24 h. CGC's were assessed for death using PI and Hoechst 33342 staining* ***(Aii).*** *Dead cells (red) were counted and expressed as percent CGC death* ***(Ai).*** *To investigate the role of Nox4 in neurotransmitter induced neuronal apoptosis, microglia were treated with the neurotransmitters as above in the presence or absence of thioridazine (1 µM) for 24 h, before media was removed and incubated with CGC's for a further 24 h to enable survival to be assessed by PI staining* ***(B).*** *To ensure that there was no direct effect of neurotransmitter's on neuronal survival, CGCs were treated with the neurotransmitters in the presence or absence of apocynin as described for 24 h. CGC death was assessed by PI staining and was expressed as percent CGC death* ***(C).*** *Data were analysed by one way ANOVA with Tukey post-hoc analysis, and comparisons were made between untreated CGCs (basal) and treatments, or as shown. *p<0.05, **p<0.01, ***p<0.001. All data are n=3. Scale bar 20 µm.*

Control MGCM had no effect on neuronal survival; however glutamate, GABA or BzATP MGCM each significantly increased neuronal death in comparison to untreated CGCs (Fig. 5.6Ai). Glutamate and apocynin MGCM significantly increased neuronal death in comparison to untreated CGCs and also when compared with glutamate MGCM treatments alone, suggesting that inhibition of glutamate - induced superoxide production in microglia promotes the release of toxic factors. However, treatment of CGCs with glutamate plus thioridazine MGCM attenuated the neuronal apoptosis seen with glutamate MGCM alone (Fig.5.5B), suggesting that glutamate induced activation of Nox4 mediates the release of neurotoxic factors. GABA MGCM significantly increased neuronal death when compared with untreated CGCs, which could be attenuated by co-treatment of microglia with GABA and apocynin (Fig. 5.6Ai). Treatment of CGCs with GABA plus thioridazine MGCM elevated neuronal death when compared with GABA MGCM treatment alone, suggesting that inhibition of Nox4 following GABA activation is neurotoxic (Fig. 5.6B). BzATP MGCM elevated neuronal death, which could be attenuated upon co-treatment of microglia with apocynin (Fig. 5.6Ai); suggesting that inhibition of BzATP mediated superoxide production attenuates the release of neurotoxic factors (Fig. 5.2B). Inhibition of microglial BzATP induced Nox4 activation with thioridazine could not protect against BzATP induced neuronal apoptosis (Fig. 5.6B).

Only direct treatment of CGCs with glutamate (1 µM) alone significantly increased neuronal death (*$p<0.05$), whilst treatment with glutamate and apocynin or GABA or BzATP in the presence or absence of apocynin had no affect on neuronal survival (Fig. 5.6C). Glutamate induced CGC death may have been a consequence of neuronal excitotoxicity (Fig. 5.6C), and as glutamate MGCM induced the same effects as direct glutamate treatment, it could be suggested that treatment of microglia with glutamate has no effect on the production or release of toxic factors, however attenuation of the NADPH oxidase following glutamate

reatment in microglia enhances the release of neurotoxic moieties. Together, these data suggest that treatment of microglia with neurotransmitters induces the release of neurotoxic or neurotrophic factors in an NADPH oxidase dependent manner, which modulates neuronal survival.

Following the findings that exposure of microglia to neurotransmitters could modulate neuronal survival in an NADPH oxidase dependent manner, it was considered important to investigate whether the release of soluble factors from microglia induced the activation of cell survival signalling pathways in CGCs. It was anticipated that this would provide further information regarding the mechanisms behind neuronal death and survival after modulation of the microglial NADPH oxidase.

Western blotting was performed to investigate phospho-Akt expression. Phosphorylated Akt promotes cell survival by inducing the phosphorylation of the pro-apoptotic protein BAD, thereby dissociating it from the Bcl-2/Bcl-x complex and inhibiting its pro-apoptotic function (Orike et al. 2001). Phospho-Akt also mediates survival through NF-κB activation, facilitating the expression of pro-survival genes (Niizuma et al. 2009). Furthermore, the neuronal Akt signalling pathway is modulated in neurons exposed to Aβ (Jimenez et al. 2011), ischaemia (Shan et al. 2009), and oxidative stress (Niizuma et al. 2009), in which these conditions promote neuronal death through the attenuation of Akt phosphorylation. In addition, TNFα favours Akt phosphorylation in fibroblasts, promoting cell survival (Chen et al. 2006). Akt is therefore implicated in neuronal survival in neurodegeneration, and its regulation by TNFα, which is modulated in microglia by neurotransmitters in an NADPH oxidase dependent manner, therefore makes this an important signalling molecule to investigate in neurons following application of neurotransmitter MGCM.

CGCs were treated with MGCM from microglia treated with the neurotransmitters glutamate (1 μM), GABA (100 μM) or BzATP (250 μM) in the presence or absence of apocynin (10 μM) for 24 h. CGCs were then lysed and subjected to Western blot analysis for phospho-Akt, and also for β-actin as a loading control (Fig. 5.7A), to enable densitometry to be performed (Fig. 5.7B).

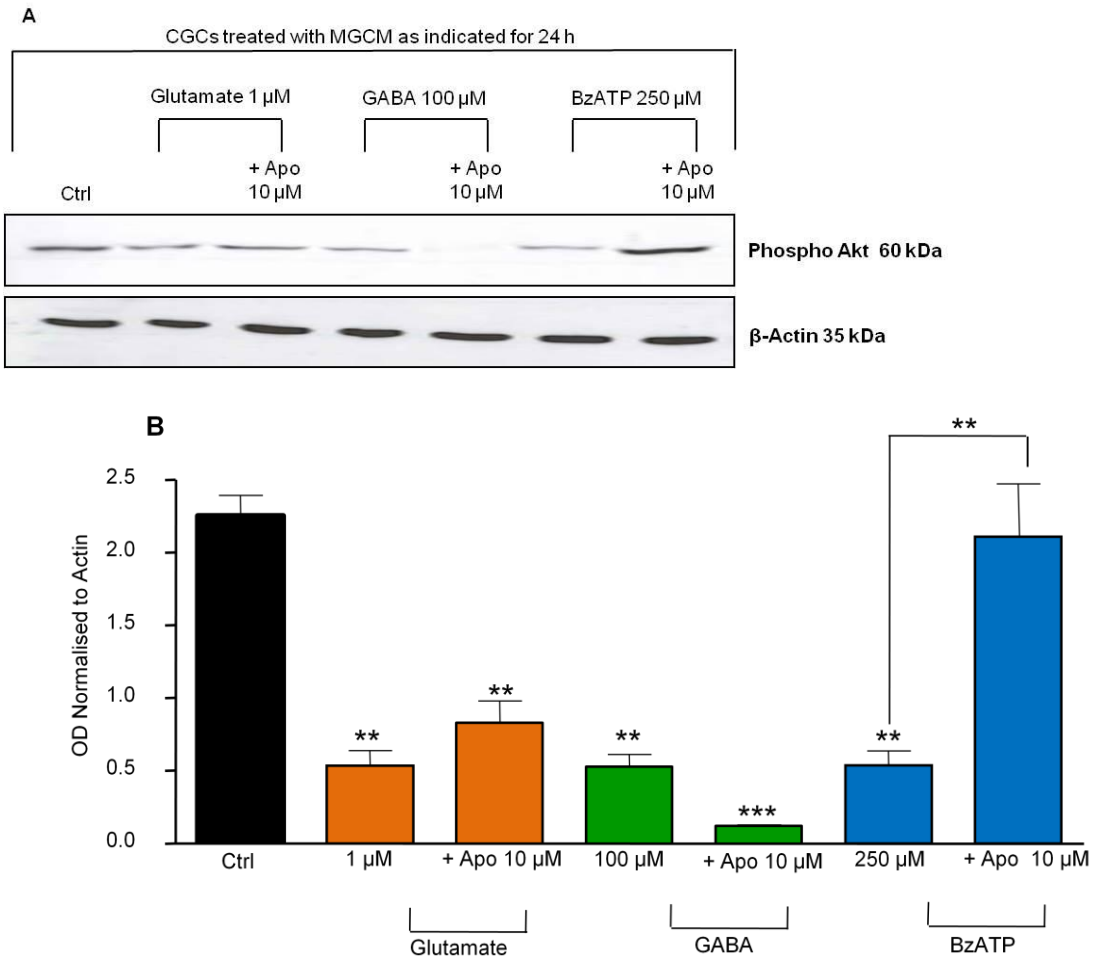

Figure 5.7 Analysis of CGC phospho-Akt expression after treatment with MGCM from microglia treated with neurotransmitters in the presence or absence of apocynin. *Microglia were treated with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence or absence of apocynin (10 µM) for 24 h. Media was then taken and used to treat CGCs for a further 24 h before neurons were lysed and lysates were run on a 12% SDS-PAGE. Protein was transferred to a PVDF membrane, which was probed for phospho-Akt expression using a goat-anti-rabbit phospho-Akt antibody (Ser473) at1:1000 overnight at 4 °C, followed by incubation with the anti-rabbit HRP secondary antibody at 1:2000 for 1 h at room temperature. Visualisation was by ECL. The membrane was then stripped and re-probed with an anti-mouse β-actin antibody at 1:2000 at room temperature for 2 h. The membrane was then incubated with the anti-mouse HRP secondary antibody at 1:1000 for 1 h at room temperature. Visualisation was by ECL* ***(A).*** *Densitometry was performed on n=3 Western blots, after normalisation to β-actin* ***(B).*** *Data were analysed by one way ANOVA and Tukey post-hoc analysis, comparing all conditions to control untreated cells, and also as shown. **p<0.01, ***p<0.001.*

Control MGCM from untreated microglia favoured Akt phosphorylation (Fig. 5.7A, B) which also correlated with enhanced CGC survival (Fig. 5.6Ai, ii). Treatment with glutamate MGCM or glutamate plus apocynin MGCM significantly decreased Akt phosphorylation in comparison with control MGCM treatments, which also correlated with neuronal death (Fig. 5.6Ai, ii). GABA MGCM also reduced Akt phosphorylation, however treatment of CGCs with GABA plus apocynin MGCM further decreased Akt phosphorylation, indicative of enhanced neuronal death (Fig. 5.7B), in contrast to the live / dead imaging analysis (Fig. 5.6). BzATP MGCM significantly attenuated Akt phosphorylation, suggestive of enhanced neuronal apoptosis, and BzATP plus apocynin MGCM significantly increased Akt phosphorylation (Fig. 5.7B), suggesting neuronal survival in line with the live/dead imaging analysis (Fig. 5.6).

These data therefore show that neuronal death can be induced by factors released from microglia treated with neurotransmitters in an NADPH oxidase dependent manner. Glutamate elevated microglial TNFα expression, which correlated with enhanced neuronal death and decreased Akt phosphorylation. Furthermore, treatment of microglia with glutamate plus apocynin further increased neuronal apoptosis and decreased Akt phosphorylation. Treatment of microglia with GABA or BzATP induced a significant increase in TNFα expression and enhanced TNFα release, which was attenuated by co-treatment with apocynin, and also correlated with an increase in neuronal death and a decrease in Akt phosphorylation, which could also be attenuated by treatment of microglia with apocynin. This therefore indicates that GABA or BzATP treatment of microglia mediates the expression and release of neurotoxic factors in an NADPH oxidase dependent manner.

The involvement of microglial neurotransmitter receptor modulation in neuronal survival as a consequence of NADPH oxidase activation was next explored. Microglia were treated with the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 μM) or the group III mGluR agonist L-AP4 (100 μM) in the presence or absence of apocynin (10 μM) for 24 h. Media was then removed, and CGC cultures were incubated with MGCM for a further 24 h before live/dead imaging was performed using PI and Hoechst 33342 (Fig. 5.8 Ai, ii). The number of PI positive cells were counted and expressed as a percentage (Fig. 5.8Ai). To ensure that the agonists or antagonists did not directly modulate of CGC survival, CGCs were treated with the receptor agonists and antagonists in the presence of apocynin as outlined above, and were assessed for neuronal death using PI and Hoechst 33342 (Fig. 5.8B).

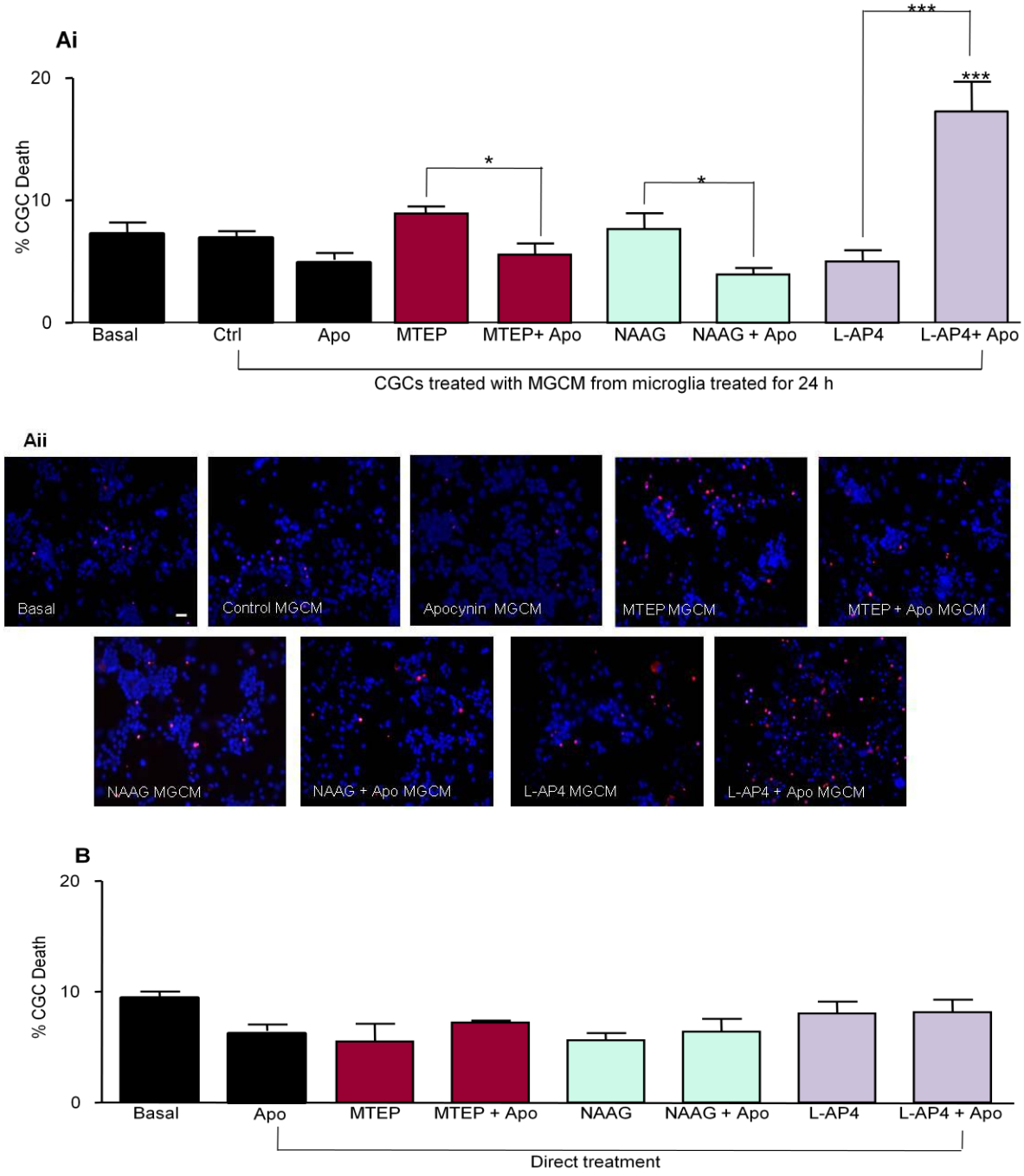

Figure 5.8 CGC death after treatment with MGCM from microglia treated with mGluR agonists or antagonists in the presence or absence of apocynin. *Microglia were treated with the group I mGluR antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 µM), or the group III mGluR agonist L-AP4 (100 µM) in the presence or absence of apocynin (10 µM) for 24 h. Culture medium was removed and placed onto CGCs grown for 7 DIV, and neurons were incubated for 24 h. CGC's were assessed for death using PI and Hoescht 33342 staining* ***(Aii).*** *Dead cells (red) were counted and expressed as percent CGC death* ***(Ai).*** *To ensure that there was no direct effect of neurotransmitter receptor induced death, CGC's were treated with the mGluR agonists and antagonists in the presence or absence of apocynin as described for 24 h. CGC death was assessed by PI staining and was expressed as percent CGC death* ***(B).*** *Data were analysed by one way ANOVA with Tukey post-hoc analysis, and comparisons were made between untreated CGC's (basal) and treatments, or as shown. *p<0.05, ***p<0.001. Data are n=3. Scale bar 20 µm.*

MGCM from microglia treated with MTEP, NAAG or L-AP4 did not modulate neuronal survival, however, co-treatment of microglia with MTEP and apocynin, or NAAG and apocynin increased neuronal survival when compared with MTEP or NAAG treatments alone (Fig. 5.8Ai), however as this reduction in neuronal death is minimal and not significant when compared with control MGCM treatments, it could be suggested that this enhanced survival may not be a consequence of attenuation of the microglial NADPH oxidase, but may be a consequence of the experimental technique employed. L-AP4 and apocynin MGCM significantly elevated neuronal death when compared with control untreated cells and also when compared with L-AP4 MGCM treatment alone (Fig. 5.8Ai). Direct treatment of CGCs with the mGluR agonists and antagonists did not modulate neuronal death (Fig. 5.8B), suggesting that the MGCM data presented in Fig. 5.8Ai is a result of the action of released factors from microglia on CGCs.

CGCs were next treated with MGCM from microglia treated with the $GABA_A$ receptor agonist muscimol (50 µM) in the presence or absence of apocynin (10 µM) for 24 h. CGC survival was assessed by live/dead imaging using PI and Hoechst 33342 as before (Fig. 5.9A, B), and cell death was expressed as a percentage (Fig. 5.9A). CGCs were also treated with muscimol (50 µM) in the presence or absence of apocynin (10 µM) directly, to determine whether there were any carry-over of effects of muscimol in the culture medium (Fig. 5.9A).

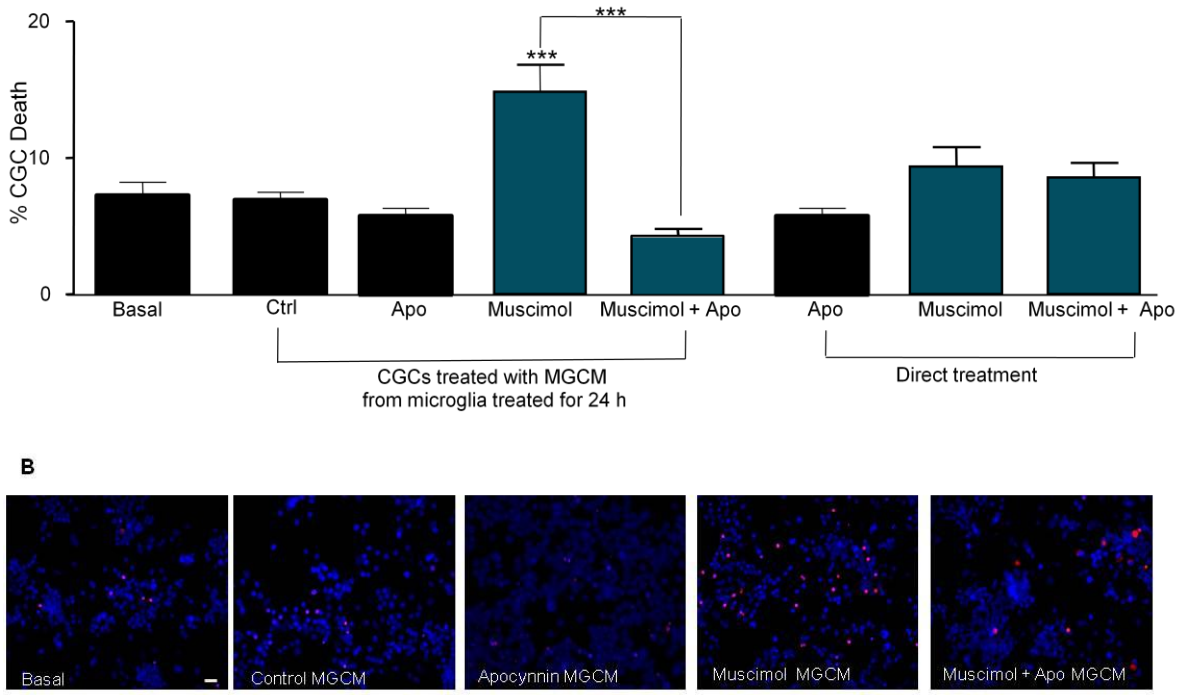

Figure 5.9 CGC death after treatment with MGCM from microglia treated with the $GABA_A$ receptor agonist in the presence or absence of apocynin. *Microglia were treated with the $GABA_A$ receptor agonist muscimol (50 µM) in the presence or absence of apocynin (10 µM) for 24 h. Culture medium was removed and placed onto CGC's grown for 7 DIV, and neurons were incubated for 24 h. CGCs were assessed for death using PI and Hoechst 33342staining* ***(A, B).*** *Dead cells (red) were counted and expressed as percent CGC death* ***(A).*** *To ensure that there was no direct effect of neurotransmitter induced death, CGCs were treated with muscimol in the presence or absence of apocynin as described for 24 h. CGC death was assessed by PI staining and was expressed as percent CGC death* ***(A).*** *Data were analysed by one way ANOVA with Tukey post-hoc analysis, and comparisons were made between control untreated cells (basal) and treatments, or as shown. ***p<0.001. Data are n=3. Scale bar 20 µm.*

Muscimol MGCM significantly increased neuronal death in an NADPH oxidase dependent manner, when compared with control untreated neurons (Fig. 5.9A). Direct treatment of CGCs with muscimol or muscimol and apocynin did not modulate neuronal survival, suggesting that muscimol promoted TNFα release from microglia in an NADPH oxidase dependent manner, which induced the neuronal death seen in Fig. 5.9A. Microglial $GABA_A$ receptor induced NADPH oxidase activation therefore contributes to neuronal death.

CGCs were next incubated with MGCM from microglia treated with the P2Y2/4 receptor agonist UTPγS (100 μM) in the presence or absence of apocynin (10 μM) for 24 h. CGCs were analysed for death using PI and Hoechst 33342 fluorescence, and PI positive CGCs were counted and expressed as a percentage of cell death (Fig. 5.10A, B). CGCs were also treated with UTPγS either alone or in the presence of apocynin directly to ensure that the effects of MGCM were not a consequence of direct neuronal actvation (Fig. 5.10A).

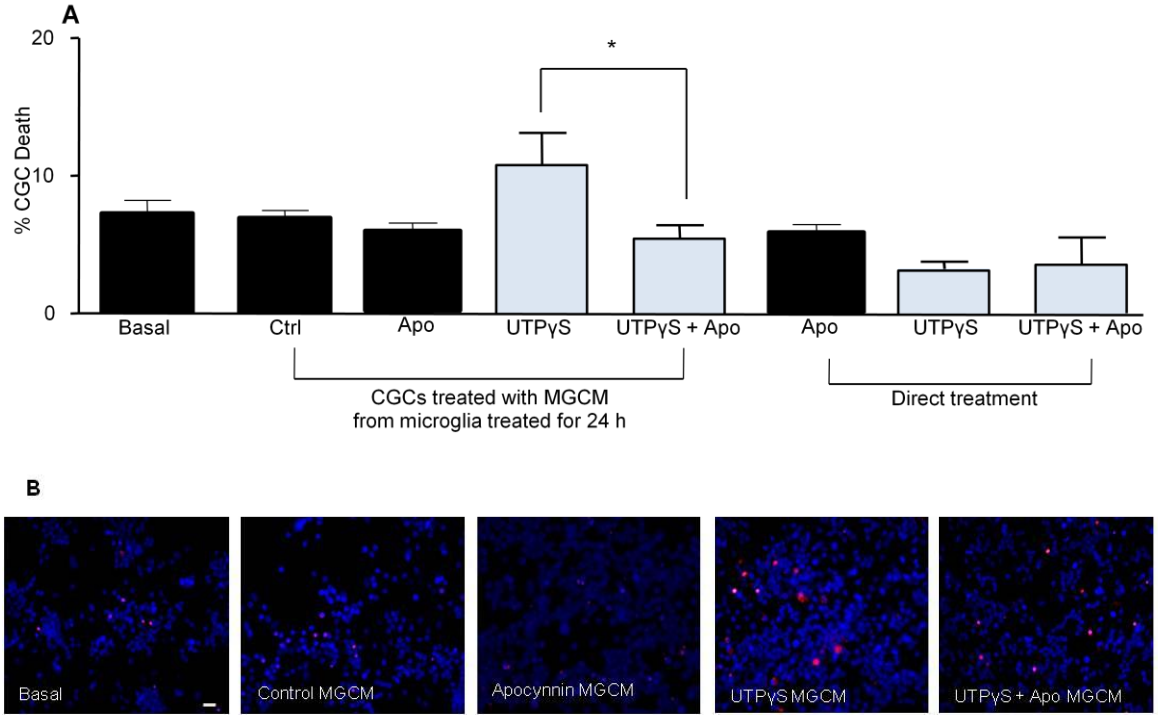

Figure 5.10 CGC death after treatment with MGCM from microglia treated with the P2Y2/4 receptor agonist in the presence or absence of apocynin. *Microglia were treated with the P2Y2/4 receptor agonist UTPγS (100 µM) in the presence or absence of apocynin (10 µM) for 24 h. Culture medium was removed and placed onto CGC's grown for 7 DIV, and neurons were incubated for 24 h. CGC's were assessed for death using PI and Hoechst 33342 staining* ***(A, B).*** *Dead cells (red) were counted and expressed as percent CGC death* ***(A).*** *To ensure that there was no direct effect of receptor induced death, CGC's were treated with UTPγS in the presence or absence of apocynin as described for 24 h. CGC death was assessed by PI staining and was expressed as percent CGC death* ***(A).*** *Data were analysed by one way ANOVA with Tukey post-hoc analysis, and comparisons were made between control untreated cells (basal) and treatments, or as shown. *p<0.05. Data are n=3. Scale bar 20 µm.*

UTPγS MGCM or UTPγS plus apocynin MGCM did not affect CGC survival when compared with untreated CGCs, however treatment of CGCs with UTPγS plus apocynin significantly enhanced neuronal survival when compared with UTPγS MGCM treatment alone (Fig. 5.10A, B). Attenuation of microglial P2Y2/4 receptor - induced superoxide production may therefore prevent the release of neurotoxic factors. Direct treatment of CGCs with UTPγS in the presence or absence of apocynin also had no effect on neuronal survival (Fig. 5.10A).

To investigate the mechanisms behind neuronal survival or death following treatment of CGCs with MGCM from microglia treated with neurotransmitter receptor agonists and antagonists, Western blot analysis was used to analyse phospho-Akt expression as before. Microglia were treated with the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 μM), the group III mGluR agonist L-AP4 (100 μM), the $GABA_A$ receptor agonist muscimol (50 μM), or the P2Y2/4 receptor agonist UTPγS (100 μM) in the presence or absence of apocynin (10 μM) for 24 h. CGCs were incubated with this MGCM for a further 24 h before lysis and Western blot analysis for phospho-Akt and β-Actin (Fig. 5.11A). Densitometry was performed to quantify phospho-Akt expression normalised to β-Actin (Fig. 5.11B).

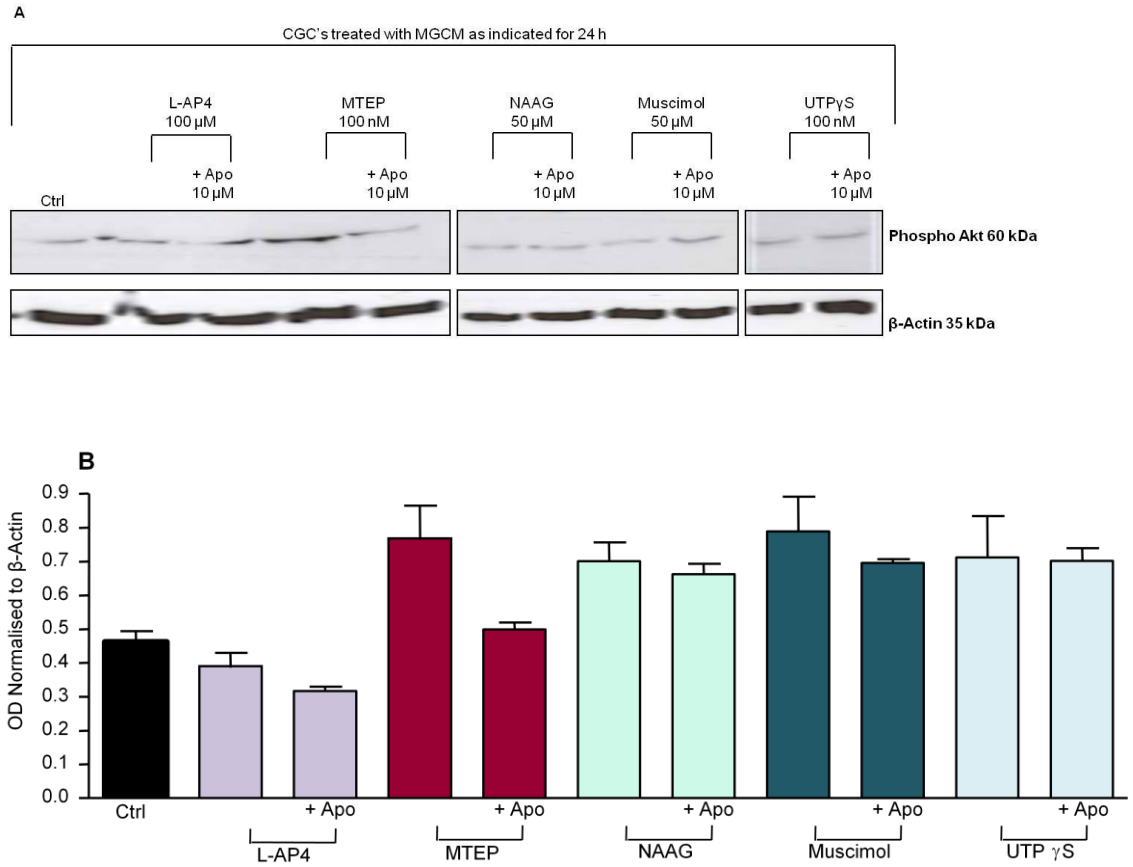

Figure 5.11 Analysis of CGC phospho-Akt expression after treatment with MGCM from microglia treated with neurotransmitter receptor agonists or antagonists in the presence or absence of apocynin. *Microglia were treated with the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 µM), the group III mGluR agonist L-AP4 (100 µM), the $GABA_A$ receptor agonist muscimol (50 µM), or the P2Y2/4 receptor agonist UTPγS (100 µM) in the presence or absence of apocynin (10 µM) for 24 h. Media was then taken and used to treat CGCs for a further 24 h before neurons were lysed and lysates were run on a 12% SDS-PAGE. Protein was transferred to a PVDF membrane, which was probed for phospho-Akt expression using a goat-anti-rabbit phospho-Akt antibody (Ser473) at 1:1000 overnight at 4 °C, followed by incubation with the anti-rabbit HRP secondary antibody at 1:2000 for 1 h at room temperature. Visualisation was by ECL. The membrane was then stripped and re-probed with an anti-mouse β-actin antibody at 1:2000 at room temperature for 2 h. The membrane was then incubated with the anti-mouse HRP secondary antibody at 1:1000 for 1 h at room temperature. Visualisation was by ECL* ***(A).*** *Densitometry was performed on n=3 Western blots, after normalisation to β-actin* ***(B).*** *Data were analysed by one way ANOVA and Tukey post-hoc analysis, comparing all conditions to control untreated cells, and also as shown. *p<0.05.*

Treatment of CGCs with MGCM from microglia treated with the group I mGluR antagonist, the group III agonist L-AP4, the mGluR3 agonist NAAG, the $GABA_A$ receptor agonist muscimol, or the P2Y2/4 recptor agonist UTPγS in the presence or absence of apocynin did not significantly increase Akt phosphorylation (Fig. 5.11A, B). These findings therefore suggest that neuronal survival following treatment of microglia with neurotransmitter receptor modulators is not mediated through an Akt dependent pathway.

As Akt phosphorylation was not significantly modulated by application of MGCM, the effects of microglial neurotransmitter receptor modulation on neuronal caspase 12 cleavage was next investigated. Caspase 12 cleavage is implicated in neuronal death in degenerative conditions. In AD, microglia release TNFα (Jekabsone et al. 2006), which acts on neuronal TNFR1 to promote ER stress, caspase 12 cleavage, and neuronal apoptosis (Shibata & Kobayashi 2008). Furthermore, TNFα and other pro-inflammatory cytokines elevate caspase 12 expression in fibroblasts, resulting in apoptosis (Kalai et al. 2003). As modulation of neurotransmitter receptors was shown to mediate neuronal survival or death in an NADPH oxidase dependent manner through the release of stable factors, it was important to investigate whether this neuronal death was a consequence of neuronal caspase 12 cleavage.

Microglia were treated with the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 μM), the group III mGluR agonist L-AP4 (100 μM), the $GABA_A$ receptor agonist muscimol (50 μM), or the P2Y2/4 receptor agonist UTPγS (100 μM) in the presence or absence of apocynin for 24 h. CGCs were incubated with MGCM for a further 24 h, and Western blot analysis was performed to detect caspase 12 and cleaved caspase 12. β-actin was used as a loading control (Fig. 5.12A). Densitometry was performed to determine the levels of cleaved caspase 12 normalised to β-actin (Fig. 5.12B).

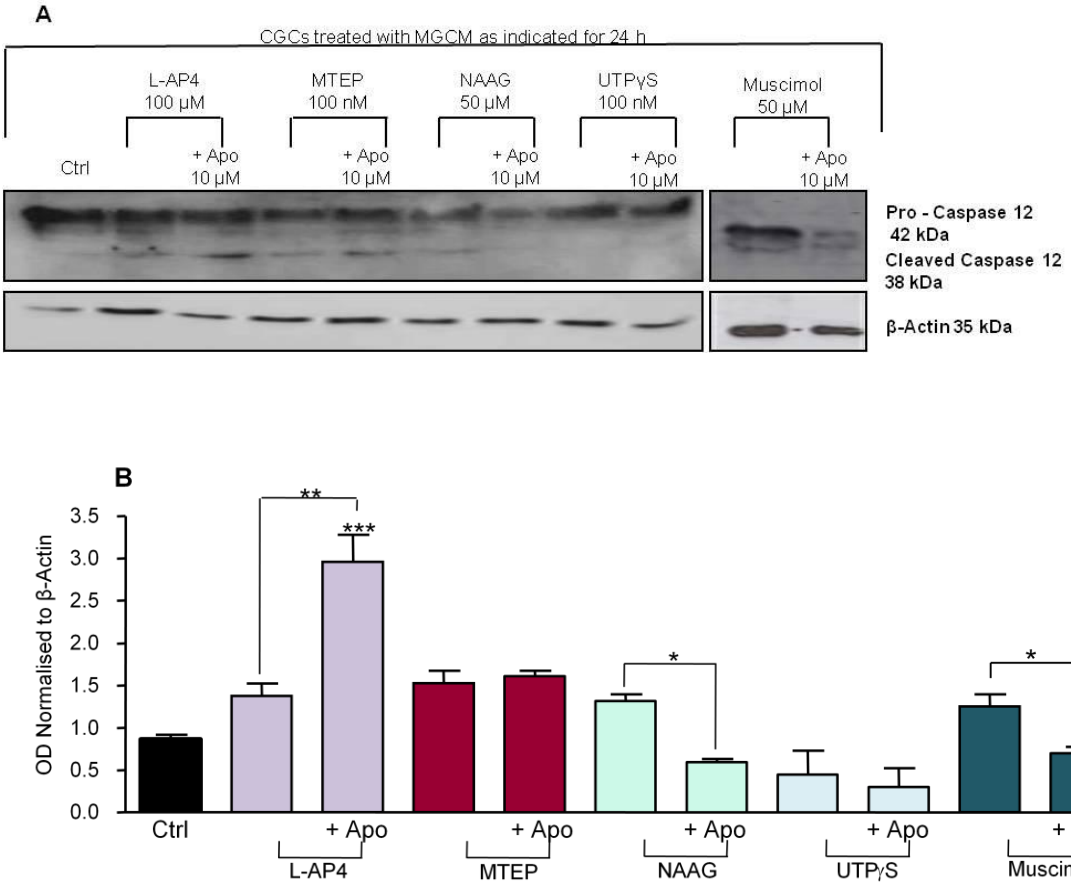

Figure 5.12 Analysis of caspase 12 cleavage in CGCs after treatment with MGCM from microglia treated with neurotransmitter receptor agonists or antagonists in the presence or absence of apocynin. *Microglia were treated with the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 µM), the group III mGluR agonist L-AP4 (1 µM), the $GABA_A$ receptor agonist muscimol (50 µM), or the P2Y2/4 receptor agonist UTPγS (100 µM) in the presence or absence of apocynin (10 µM) for 24 h. Media was then taken and used to treat CGC's for a further 24 h before neurons were lysed and lysates were run on a 12% SDS-PAGE. Protein was transferred to a PVDF membrane, which was probed for cleaved caspase 12 expression using a goat-anti-rabbit caspase 12 antibody at1:1000 overnight at 4 °C, followed by incubation with the anti-rabbit HRP secondary antibody at 1:1000 for 1 h at room temperature. Visualisation was by ECL. The membrane was then stripped and re-probed with an anti-mouse β-actin antibody at 1:2000 at room temperature for 2 h. The membrane was then incubated with the anti-mouse HRP secondary antibody at 1:1000 for 1 h at room temperature. Visualsation was by ECL* ***(A).*** *Densitometry was performed on n=3 Western blots, after normalisation to β-actin* ***(B).*** *Data were analysed by one way ANOVA and Tukey post-hoc analysis, comparing all conditions to control untreated cells, and also as shown. *p<0.05, **p<0.01, ***p<0.001.*

Treatment of CGCs with MGCM from microglia treated with the neurotransmitter receptor agonists or antagonists modulated caspase 12 cleavage in line with the live/dead analysis. Treatment of CGCs with MGCM from microglia treated with the mGluR group III agonist L-AP4 did not affect caspase 12 cleavage when compared with control untreated cells, however, treatment of CGCs with MGCM from microglia treated with L-AP4 and apocynin significantly increased caspase 12 cleavage when compared with control untreated cells and also when compared with L-AP4 MGCM treatment alone (Fig. 5.12A, B), which agreed with the live/dead analysis whereby L-AP4 and apocynin MGCM significantly increased neuronal death (Fig. 5.8Ai). Treatment of CGCs with MTEP MGCM or MTEP plus apocynin MGCM had little effect on caspase 12 cleavage (Fig. 5.12B). NAAG MGCM did not increase neuronal caspase 12 cleavage, however co-treatment of microglia with NAAG and apocynin significantly decreased neuronal caspase 12 cleavage (Fig. 5.12A, B), suggesting that inhibition of NAAG induced superoxide production in microglia is neuroprotective, in agreement with the live/dead analysis (Fig. 5.8A). UTPγS MGCM or UTPγS plus apocynin MGCM did not significantly alter neuronal caspase 12 cleavage, however, caspase 12 cleavage mediated by treatment of CGCs with muscimol MGCM could be attenuated by apocynin (Fig. 5.12A, B), suggesting that muscimol induced superoxide production may enhance neuronal death in a caspase 12 dependent manner.

The mechanisms of neuronal death or survival following exposure of CGCs to MGCM from microglia treated with neurotransmitter receptor agonists or antagonists in the presence or absence of apocynin was further explored by investigating neuronal TNFR1 expression. TNFR1 plays an important role in neuronal death, inducing a cascade of events leading to apoptosis after exposure to TNFα (Yang et al. 2002), and as mentioned previously, mediates apoptosis through initiating caspase 12 cleavage (Shibata & Kobayashi 2008), and also a caspase 3 death cascade (Harry et al. 2008). TNFR1 expression is modulated by exposure of

neurons to activating stimuli, and TNFR1 is shed from neurons after exposure to TNFα, which promotes neuroprotection by reducing the bioavailability of TNFα to membrane bound TNFR's (Bartsch et al. 2010). Furthermore, TNFR1 expression is modulated in a time dependent manner in *in vivo* models of ischaemia, which correlates with increased neuronal death during high levels of TNFR1 expression (Botchkina et al. 1997). The suggestions therefore that TNFR1 expression may be regulated by TNFα levels and that TNFα binding to TNFR1 is neurotoxic, lead to an investigation into the expression of this receptor on CGCs following exposure to MGCM from microglia treated with receptor agonists or antagonists in the presence or absence of apocynin.

CGCs were treated with MGCM from microglia treated with the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 μM), the group III agonist L-AP4 (100 μM), the $GABA_A$ receptor agonist muscimol (50 μM), or the P2Y2/4 receptor agonist UTPγS (100 μM) in the presence or absence of apocynin (10 μM), for 24 h. CGCs were then lysed and subjected to Western blot analysis for TNFR1 and β-actin expression (Fig. 5.13A). Densitometry was performed to semi-quantify TNFR1 expression after normalisation to β-actin (Fig. 5.13B).

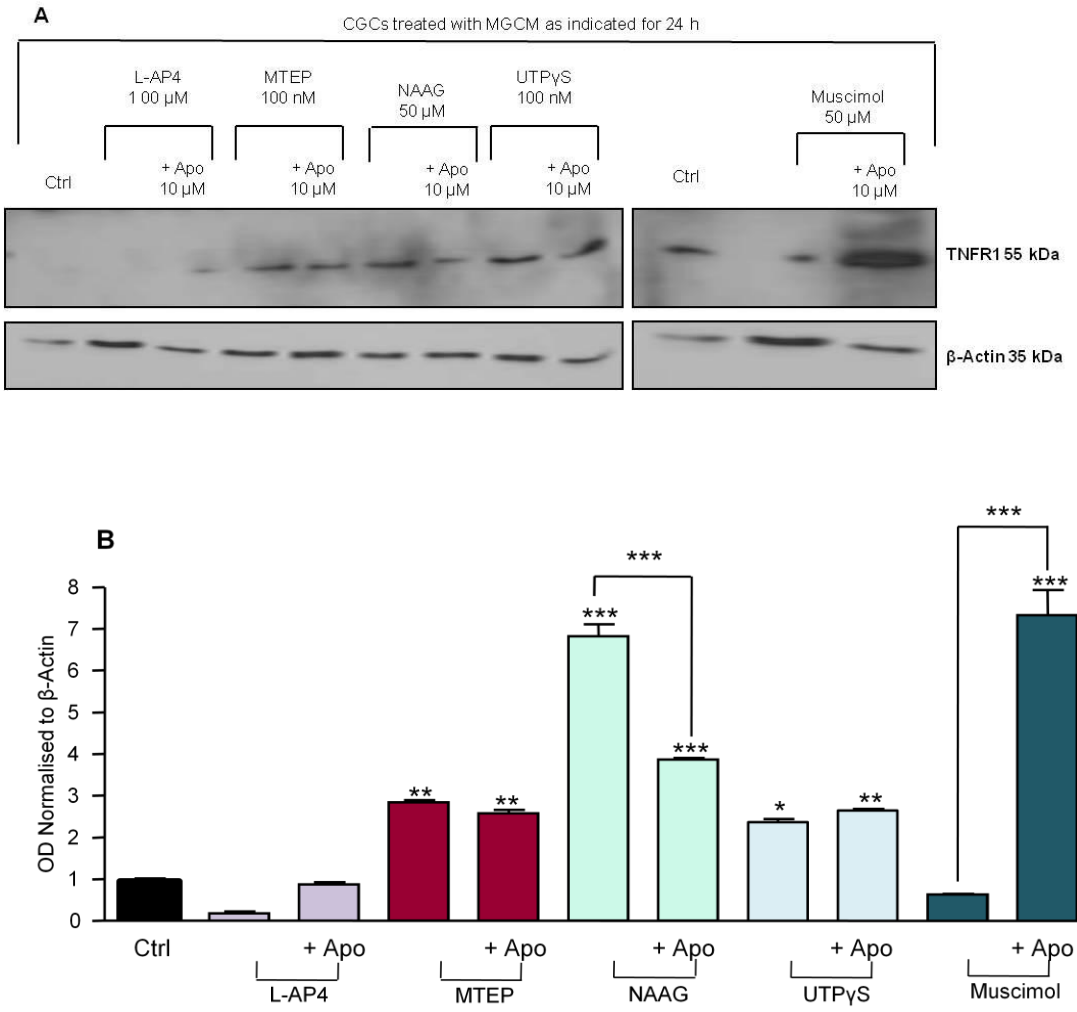

Figure 5.13 Analysis of TNFR1 expression in CGCs after treatment with MGCM from microglia treated with neurotransmitter receptor agonists or antagonists in the presence or absence of apocynin. *Microglia were treated with the mGluR group I antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 µM), the group III mGluR agonist L-AP4 (100 µM), the $GABA_A$ receptor agonist muscimol (50 µM), or the P2Y2/4 receptor agonist UTPγS (100 µM) in the presence or absence of apocynin (10 µM) for 24 h. Media was then taken and used to treat CGCs for a further 24 h before neurons were lysed and lysates were run on a 12% SDS-PAGE. Protein was transferred to a PVDF membrane, which was probed for TNFR1 expression using a goat-anti-rabbit TNFR1 antibody at1:1000 overnight at 4 °C, followed by incubation with the anti-rabbit HRP secondary antibody at 1:2000 for 1 h at room temperature. Visualisation was by ECL. The membrane was then stripped and re-probed with an anti-mouse β-actin antibody at 1:2000 at room temperature for 2 h. The membrane was then incubated with the anti-mouse HRP secondary antibody at 1:1000 for 1 h at room temperature. Visualisation was by ECL* ***(A).*** *Densitometry was performed on n=3 Western blots, after normalisation to β-actin* ***(B).*** *Data were analysed by one way ANOVA and Tukey post-hoc analysis, comparing all conditions to control untreated cells, and also as shown. *p<0.05, **p<0.01, ***p<0.001.*

Treatment of CGCs with MGCM from microglia treated with L-AP4 or L-AP4 plus apocynin did not significantly affect TNFR1 expression (Fig. 5.13A, B). MTEP or MTEP plus apocynin MGCM both significantly increased neuronal TNFR1 expression (Fig. 5.13A, B). Treatment of CGCs with NAAG MGCM significantly enhanced TNFR1 expression, which could be attenuated by co-treatment of microglia with NAAG plus apocynin (Fig. 5.13A, B). This correlates with the caspase 12 Western blot analysis (Fig. 5.12A, B), and also with the live/dead analysis (Fig. 5.8A), which showed elevated cleaved caspase 12 expression and neuronal death which could be attenuated by apocynin, suggesting that microglial mGluR3 induced NADPH oxidase activation is neurotoxic. Treatment of CGCs with UTPγS or UTPγS plus apocynin MGCM significantly increased TNFR1 expression (Fig. 5.13A, B). In addition, muscimol MGCM had no effect on TNFR1 expression; however co-treatmemt of microglia with muscimol and apocynin enhanced neuronal TNFR1 expression (Fig. 5.13A, B). This is in contrast to live/dead analysis (Fig. 5.9A) and caspase 12 expression (Fig. 5.12A, B), showing that muscimol MGCM induces neuronal death and elevated caspase 12 cleavage in an NADPH oxidase dependent manner.

Preliminary investigations were also conducted into neuronal survival following treatment of CGCs with MGCM from microglia treated with the iGluR agonists and antagonists. Microglia were treated with the NMDA receptor agonist NMDA (100 µM) or the antagonist MK-801 (10 µM), either alone, in combination or in combination with apocynin (10 µM); or were treated with the AMPA receptor agonist QA (10 µM), or the antagonist CNQX (10 µM), either alone, in combination, or in combination with apocynin (10 µM) for 24 h. CGCs were incubated with this MGCM, and were assessed for cell death following fixation and incubation with Hoechst 33342, after which the number of pyknotic nuclei (Fig. 5.14B) were counted and expressed as a percentage of neuronal apoptosis (Fig. 5.14A).

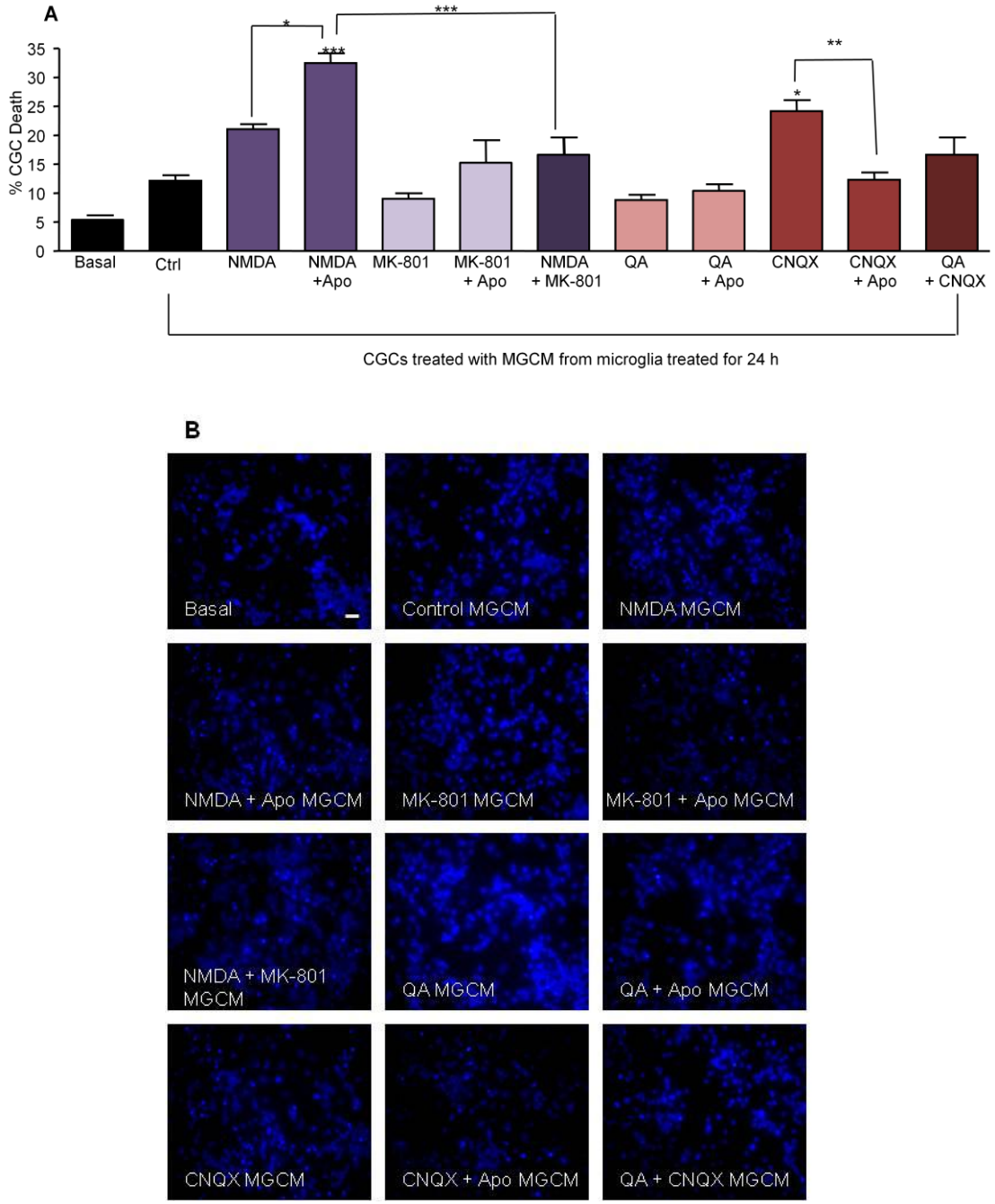

Figure 5.14 Modulation of microglial iGluRs affects neuronal survival. *Primary microglia were treated with the NMDA receptor agonist NMDA (100 µM), or the antagonist MK-801 (10 µM), either alone, in combination, or in combination with apocynin (10 µM); or were treated with the AMPA receptor agonist QA (10 µM), or the antagonist CNQX (10 µM), either alone, in combination, or in combination with apocynin (10 µM). Media was taken after 24 h and used to treat CGCs for a further 24 h. Cells were assessed for death using Hoescht 33342 staining* ***(B)****, in which the number of pyknotic nuclei were counted and expressed as a percentage of apoptotic neurons* ***(A).*** *Data were analysed using a one way ANOVA with post hoc analysis. *p<0.05, **p<0.01, ***p<0.001. Data are n=3. Scale bar 20 µm.*

NMDA plus apocynin MGCM significantly elevated neuronal death in comparison to control untreated cells and also when compared with CGCs treated with NMDA and MK-801 MGCM (Fig. 5.14A). This suggests that superoxide production from NMDA receptor activation promotes the production of protective factors. Treatment of microglia with the AMPA receptor antagonist also increased neuronal death, which was attenuated by co-treatment with apocynin (Fig. 5.14A), suggesting that CNQX induced superoxide production is neurotoxic. However, as controls to investigate the direct effects of NMDA or AMPA on CGC survival were not conducted, and as the direct application of NMDA would almost certainly induce excitotoxic death to CGCs, these findings can only be considered a preliminary investigation.

In summary, these data show that activation or inhibition of the microglial neurotransmitter receptors can modulate neuronal survival in an NADPH oxidase dependent manner. Treatment of microglia with MTEP induced a slight increase in neuronal death, which could be attenuated by apocynin inhibition of microglial MTEP induced superoxide production. NAAG treatment of microglia induced an increase in neuronal death which could be attenuated by apocynin, which correlated with a decrease in caspase 12 cleavage and TNFR1 expression upon treatment of CGCs with NAAG plus apocynin MGCM, suggesting that inhibition of NAAG induced NADPH oxidase activation is protective. In contrast, L-AP4 plus apocynin MGCM significantly increased neuronal death and also elevated caspase 12 cleavage, suggesting that the inhibition of microglial L-AP4 induced superoxide production is neurotoxic. Muscimol MGCM increased CGC death, which was attenuated by co-treatment of microglia with apocynin, suggesting that muscimol induced the release of toxic factors, and indeed, was shown to up-regulate TNFα release in an NADPH oxidase dependent manner. Neuronal death was not modulated significantly by treatment with UTPγS MGCM. however UTPγS plus apocynin MGCM enhanced neuroprotection. These data therefore

indicate that superoxide induced production of released factors from microglia can affect neuronal survival. The effects of these factors on the survival of damaged neurons was investigated next.

5.2.3 MGCM from microglia treated with neurotransmitters and receptor agonists or antagonists modulates neuronal survival after treatment with staurosporine, a model of neuronal apoptosis, in an NADPH oxidase dependent manner

Following the findings that modulation of the microglial NADPH oxidase with neurotransmitter receptor agonists or antagonists influenced the survival of healthy neurons, it was important to investigate the protective or toxic effects of activation of the microglial NADPH oxidase by neurotransmitters and receptor modulation on damaged neurons. A model of apoptosis was used, in which CGCs were treated with staurosporine (Sts, 0.5 µM) for 4 h, which was shown to induce a high level of neuronal death by PI and Hoechst imaging. Staurosporine has been used as an apoptotic inducer in many studies of neuronal apoptosis. It is a broad range protein kinase inhibitor that promotes apoptosis by attenuating cellular activity (Gani & Engh 2010). Furthermore, staurosporine has been used as an inducer of apoptosis in MGCM studies to investigate the role of released factors from microglia on CGC survival (Eleuteri et al. 2008), which lead to the use of staurosporine in this manner here.

Initally, microglia were treated with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence or absence of apocynin for 24 h (Fig.5.15A, B) or for 8 h, 12 h or 24 h for a time course analysis (Fig. 5.15Ci, ii, iii). CGCs were then treated with Sts (0.5 µM) in the presence of MGCM for 4 h, and cell death was assessed by PI and Hoechst imaging, and was expressed as a percentage of apoptosis.

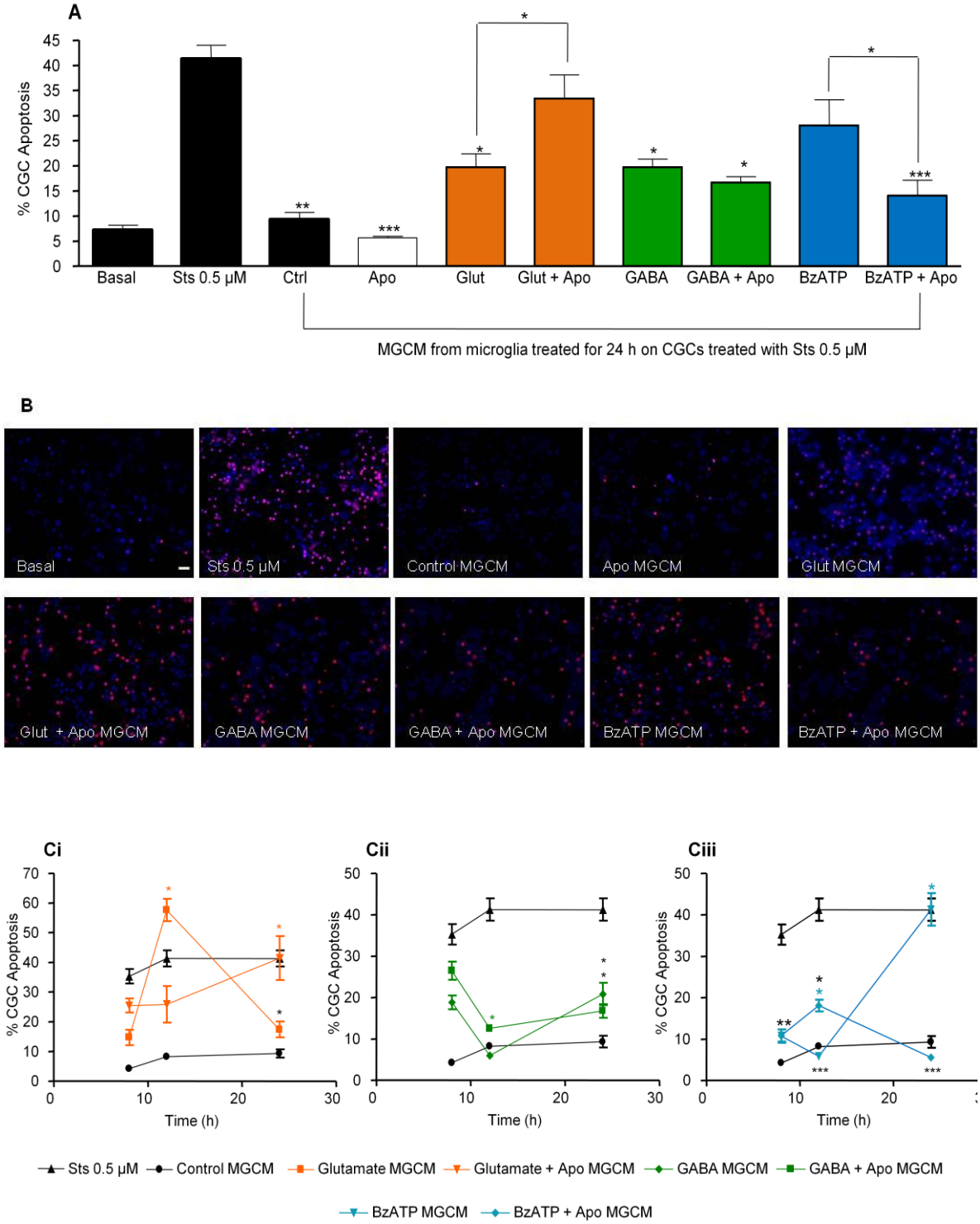

Figure 5.15 legend overleaf

Figure 5.15 Neuronal apoptosis after treatment of CGCs Sts and MGCM from microglia treated with neurotransmitters in the presence or absence of apocynin. *Microglia were either left untreated, treated with apocynin (10 µM) alone or were treated with the neurotransmitters glutamate (1 µM), GABA (100 µM) or BzATP (250 µM) in the presence or absence of apocynin (10 µM) for 24 h before media was taken and added to CGCs treated with Sts (0.5 µM) for 4 h, after which cells were incubated with PI and Hoechst 33342 and dead cells were counted to determine the percentage neuronal death* ***(A, B).*** *Microglia were also treated with the neurotransmitters glutamate (1 µM) or glutamate plus apocynin for 8 h, 12 h or 24 h* ***(Ci)****, GABA (100 µM) or GABA plus apocynin for 8 h, 12 h or 24 h* ***(Cii)****, or BzATP or BzATP plus apocynin for 8 h, 12h, or 24 h* ***(Ciii)****, before media was taken and added to CGC's treated with Sts. CGC's were treated for 4 h and cell death was also assessed by PI and Hoechst 33342 staining to determine the percentage of CGC apoptosis. Data were analysed by one way ANOVA and Tukey post hoc analysis, in which all treatments were compared with Sts treatment alone, and also as indicated. *p<0.05, **p<0.01, ***p<0.001. Data are n=3. Scale bar 20 µm.*

Treatment of CGCs with Sts (0.5 μM) for 4 h induced ~45% neuronal death, which was significantly attenuated by treatment of Sts treated CGCs with control MGCM (Fig. 5.15A), in agreement with Eleuteri et al. (2008); suggesting that resting microglia express trophic factors that aid neuronal survival after injury. Glutamate MGCM provided protection against Sts induced neuronal apoptosis in an NADPH oxidase dependent manner, as treatment of CGCs with glutamate plus apocynin MGCM could not attenuate neuronal death following exposure to Sts (Fig. 5.15A). GABA or GABA plus apocynin MGCM both afforded similar levels of neuroprotection against Sts induced neuronal damage (Fig. 5.15A), suggesting that GABA induced activation of the microglial NADPH oxidase may not be involved in neuronal survival after injury. BzATP MGCM could not protect Sts exposed CGCs from apoptosis when compared with CGCs treated with Sts alone, however, exposure of CGCs to BzATP plus apocynin MGCM in the presence of Sts was protective in comparison to BzATP MGCM treatment and also when compared with Sts treatment alone (Fig. 5.15A). BzATP may therefore induce the release of neurotoxic factors from microglia in an NADPH oxidase dependent manner, and contribute to neuronal death following damage.

MGCM from microglia treated with glutamate for 8 h did not offer protection against Sts induced apoptosis (Fig. 5.15Ci), however, at 12 h treatment of microglia with glutamate, MGCM increased Sts induced apoptosis when compared with Sts treatments alone and also in comparison with 24 h treatments. Acute exposure of microglia to glutamate may induce an increase in NADPH oxidase activity and subsequent neuronal damage, whereas chronic exposure is less damaging. MGCM from microglia treated with GABA for 12 h induced a significant level of protection against neuronal apoptosis when compared with Sts treatment alone (Fig. 5.15Cii). In addition, MGCM from microglia treated with GABA plus apocynin for 12 h significantly decreased neuronal apoptosis when compared with GABA MGCM treatment or Sts treatment alone (Fig. 5.15Cii), suggesting that attenuation of GABA induced

NADPH oxidase activation may inhibit the release of neurotoxic substances. MGCM from microglia treated with BzATP for 12 h significantly decreased neuronal apoptosis (Fig. 5.15Ciii), suggesting that acute exposure of microglia to BzATP may be neuroprotetcive, whereas chronic exposure of microglia to BzATP is not protective against neuronal damage.

The same experiment was then performed to investigate the effects of MGCM from microglia treated with mGluRs in the presence or absence of apocynin on CGC survival after treatment with Sts. Microglia were treated with the group I mGluR antagonist MTEP (100 µM), the mGluR3 agonist NAAG (50 µM), or the group III agonist L-AP4 (100 µM) in the presence or absence of apocynin (10 µM) for 24 h before incubation with CGCs for 4 h with Sts (Fig. 5.16A, B). A time-course analysis was also performed, in which microglia were treated with MTEP (Fig. 5.16Ci), NAAG (Fig. 5.16Cii), or L-AP4 (Fig. 5.16Ciii) in the presence or absence of apocynin for 8 h, 12 h or 24 h before media was taken and applied to CGCs co-treated with Sts for 4 h. Neuronal survival was analysed by PI and Hoechst imaging.

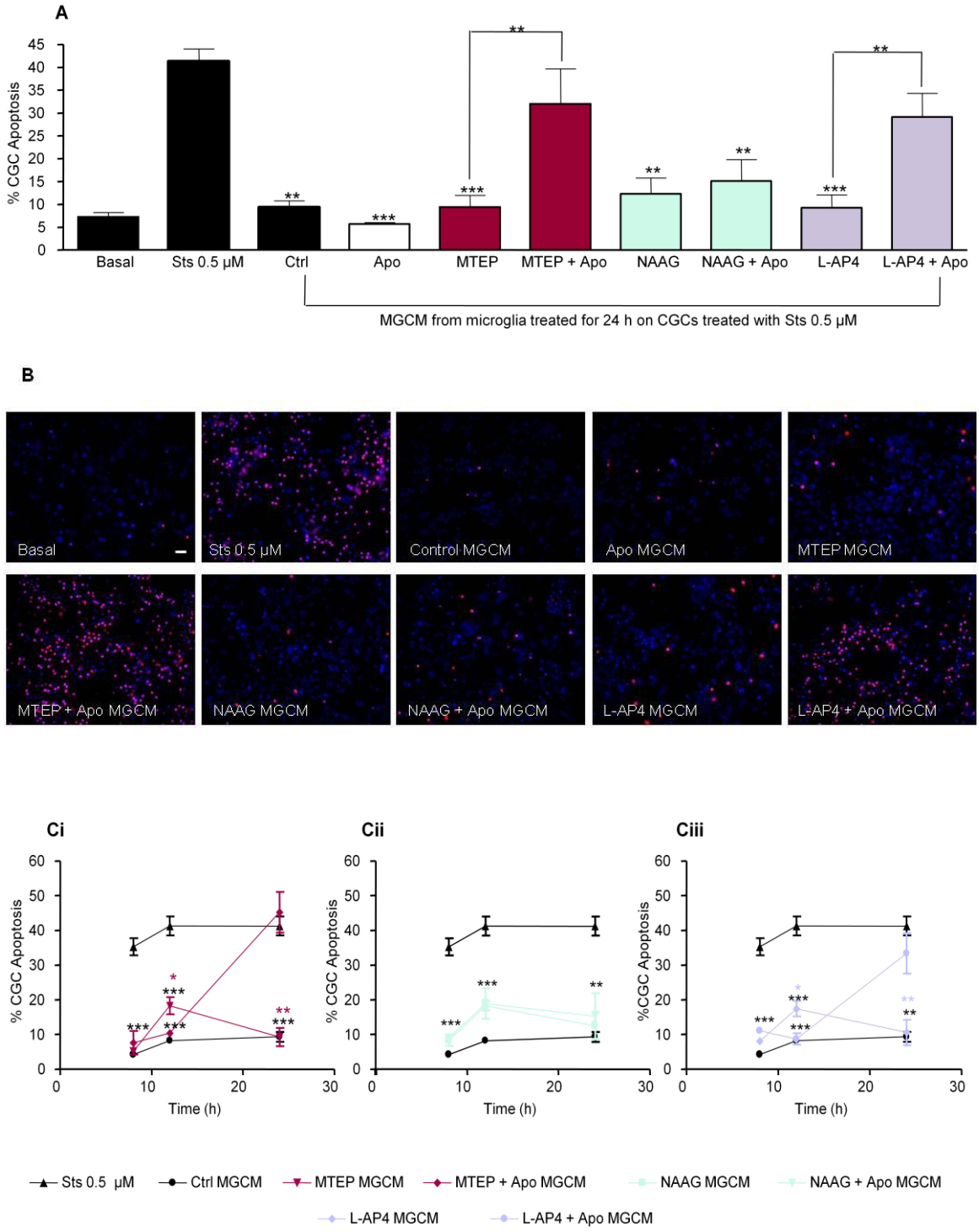

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Figure 5.16 Neuronal apoptosis after treatment of CGCs Sts and MGCM from microglia treated with mGluR receptor agonists or antagonists in the presence or absence of apocynin. *Microglia were either left untreated, treated with apocynin (10 µM) alone or were treated with the group I mGluR antagonist MTEP (100 nM), the mGluR3 agonist NAAG (50 µM) or the group III mGluR agonist L-AP4 (100 µM) in the presence or absence of apocynin for 24 h before media was taken and added to CGCs treated with Sts (0.5 µM) for 4 h, after which cells were indubated with PI and hoescht and dead cells were counted to determine the percentage neuronal death* ***(A, B).*** *Microglia were also treated with the group I mGluR antagonist MTEP (100 nM) plus apocynin for 8 h, 12 h or 24 h* ***(Ci)****, the mGluR3 agonist NAAG (50 µM) in the presence or absence of apocynin for 8 h, 12 h or 24 h* ***(Cii)****, or the group III mGluR agonist L-AP4 (100 µM) in the presence or absence of apocynin for 8 h, 12h, or 24 h* ***(Ciii)****, before media was taken and added to CGC's treated with Sts. CGCs were treated for 4 h and cell death was also assessed by PI and hoescht staining to determine the percentage of CGC apoptosis. Data were analysed by one way ANOVA and Tukey post hoc analysis, in which all treatments were compared with Sts treatment alone, and also as indicated. *p<0.05, **p<0.01, ***p<0.001. Data are n=3. Scale bar 20 µm.*

MTEP or L-AP4 MGCM significantly attenuated Sts induced neuronal apoptosis when compared with Sts treatment alone (Fig. 5.16A). Furthermore, attenuation of the microglial NADPH oxidase activation following MTEP or L-AP4 treatment with apocynin inhibited the protective effect of MTEP or L-AP4 MGCM on the survival of damaged neurons (Fig. 5.16A), suggesting that the production of superoxide in microglia following MTEP or L-AP4 treatment for 24 h may induce the release of neuroprotective factors. In contrast, NAAG MGCM or NAAG plus apocynin MGCM both significantly attenuated Sts induced neuronal death, suggesting that the NADPH oxidase is not implicated in microglial mGluR3 mediated neuroprotection (Fig. 5.16A).

MTEP MGCM from microglia treated for 12 h significantly attenuated Sts induced neuronal apoptosis when compared with Sts treatment alone (Fig. 5.16Ci). MTEP and apocynin MGCM increased Sts induced neuronal death in comparison to MTEP MGCM treatment, however death was significantly reduced when compared with Sts treatment alone, suggesting that after 12 h incubation, MTEP mediates NADPH oxidase activation and production of neuroprotective factors (Fig. 5.16Ci). NAAG MGCM from microglia treated for 8 h, 12 h or 24 h all significantly attenuated Sts induced neuronal apoptosis when compared with treatment of CGCs with Sts alone (Fig. 5.16Cii). This effect was not dependent on modulation of the microglial NADPH oxidase, as co-treatment of microglia with NAAG and apocynin did not affect CGC survival following incubation with MGCM at any time-point (Fig. 5.16Cii). L-AP4 MGCM following treatment of microglia for 12 h was neuroprotective against Sts treatment, however this could not be modulated by inhibition of the microglial NADPH oxidase in the same way as incubation of microglia with L-AP4 for for 24 h (Fig. 5.16Ciii), thereby suggesting that activation of the microglial NADPH oxidase following activation of the microglial group III mGluRs occurs at 24 h and at this time point, neuroprotective factors are released (Fig. 5.16Ciii).

The role of microglial $GABA_A$ induced NADPH oxidase activity on neuronal survival after treatment with Sts was next investigated (Fig. 5.17). Microglia were treated with the $GABA_A$ receptor agonist muscimol (50 μM) in the presence or absence of apocynin (10 μM) for 24 h before MGCM was taken and added to CGCs treated with Sts for a further 4 h (Fig, 5.17A). Neuronal survival was assessed by PI and Hoechst imaging (Fig. 5.17A, B). To determine whether the production of toxic or protective factors from microglia treated with muscimol was time dependent, a time course analysis was performed (Fig. 5.17C), in which microglia were treated with muscimol in the presence of apocynin for 8 h, 12 h or 24 h and MGCM was taken and added to CGCs treated with Sts for 4 h.

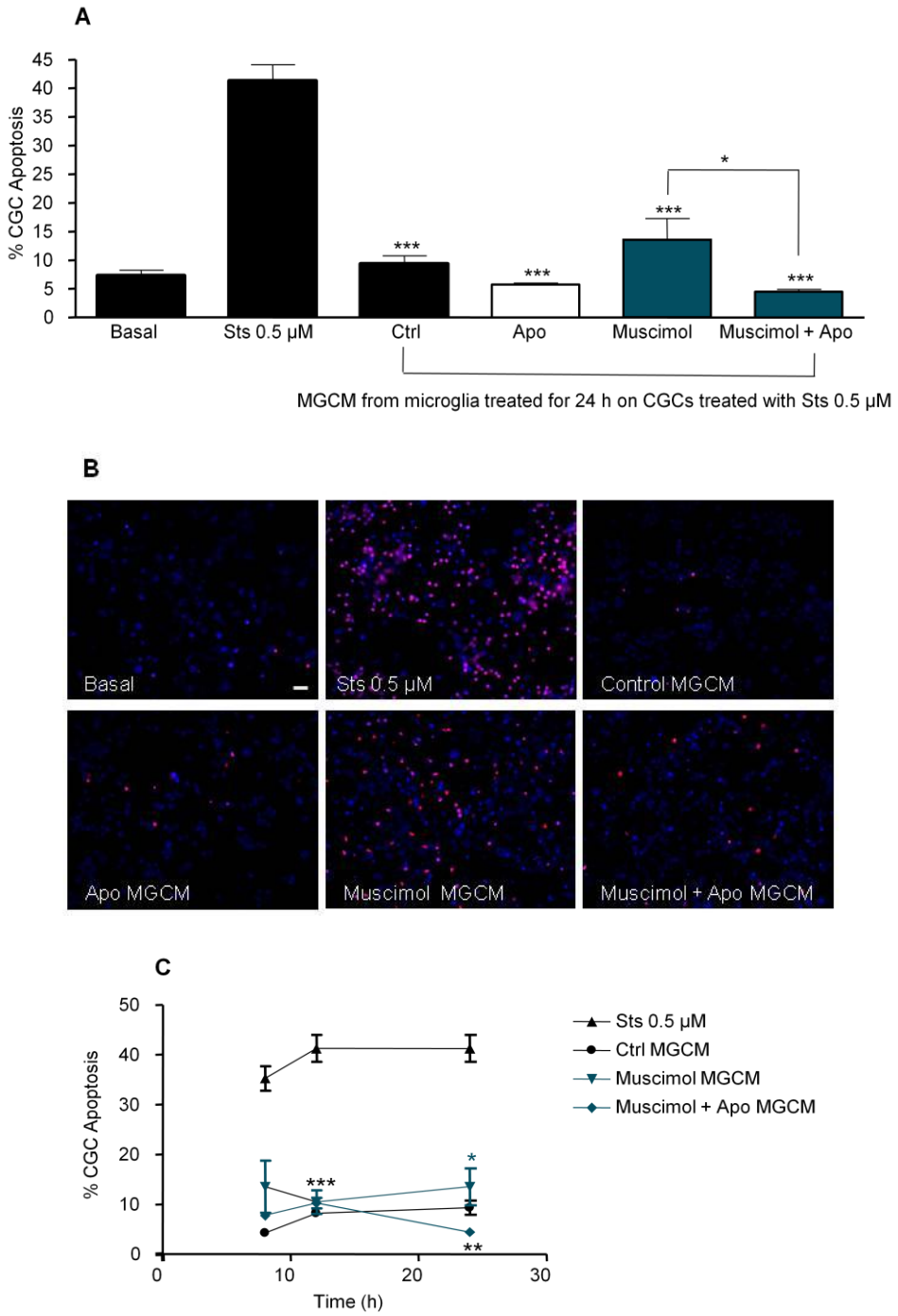

Figure 5.17Neuronal apoptosis after treatment of CGCs with Sts and MGCM from microglia treated with the $GABA_A$ receptor agonist in the presence or absence of apocynin. *Microglia were either left untreated, treated with apocynin (10 µM) alone or were treated with $GABA_A$ receptor agonist muscimol (50 µM) in the presence or absence of apocynin for 24 h before media was taken and added to CGCs treated with Sts (0.5 µM) for 4 h, after which cells were indubated with PI and Hoechst 33342 and dead cells were counted to determine the percentage neuronal death* ***(A, B).*** *Microglia were also treated with the $GABA_A$ receptor agonist muscimol in the presence or absence of apocynin for 8 h, 12h, or 24 h* ***(C)****, before media was taken and added to CGC's treated with Sts. CGCs were treated for 4 h and cell death was also assessed by PI and Hoechst 33342 staining to determine the percentage of CGC apoptosis. Data were analysed by one way ANOVA and Tukey post hoc analysis, in which all treatments were compared with Sts treatment alone, and also as indicated. *p<0.05, **p<0.01, ***p<0.001. Data are n=3. Scale bar 20 µm.*

Muscimol MGCM (from microglia treated for 24 h) significantly attenuated Sts induced neuronal apoptosis when compared with Sts treatment alone (Fig. 5.17A). This neuronal survival could be further enhanced by treatment of Sts treated CGCs with muscimol plus apocynin MGCM, suggesting that inhibition of muscimol induced NADPH oxidase activity prevents the release of neurotoxic factors and is neuroprotective (Fig. 5.17A). A time-course analysis showed that at 8 h and 12 h treatment of microglia with muscimol or muscimol plus apocynin, MGCM offered significant neuroprotection, however there was no difference between the effects of muscimol MGCM or muscimol plus apocynin MGCM (Fig. 5.17C), suggesting that only after 24 h, activation of the microglial $GABA_A$ receptor induces NADPH oxidase derived neuroprotection.

The role of microglial P2Y2/4 receptor induced NADPH oxidase activity on neuronal suurvival following treatment with Sts was also investigated (Fig. 5.18). Microglia were treated with the P2Y2/4 receptor agonist UTPγS (100 μM) in the presence or absence of apocynin for 24 h before MGCM was taken and added to CGCs treated with Sts for a further 4 h (Fig, 5.18A). Neuronal survival was assessed by PI and Hoechst imaging and was expressed as a percentage of apoptotic neurons (Fig. 5.18A, B). To determine whether the production of toxic or protective factors from microglia treated with UTPγS was time dependent, a time course analysis was performed (Fig. 5.18C), in which microglia were treated with UTPγS in the presence of apocynin for 8 h, 12 h or 24 h and MGCM was taken and added to CGCs treated with Sts for 4 h.

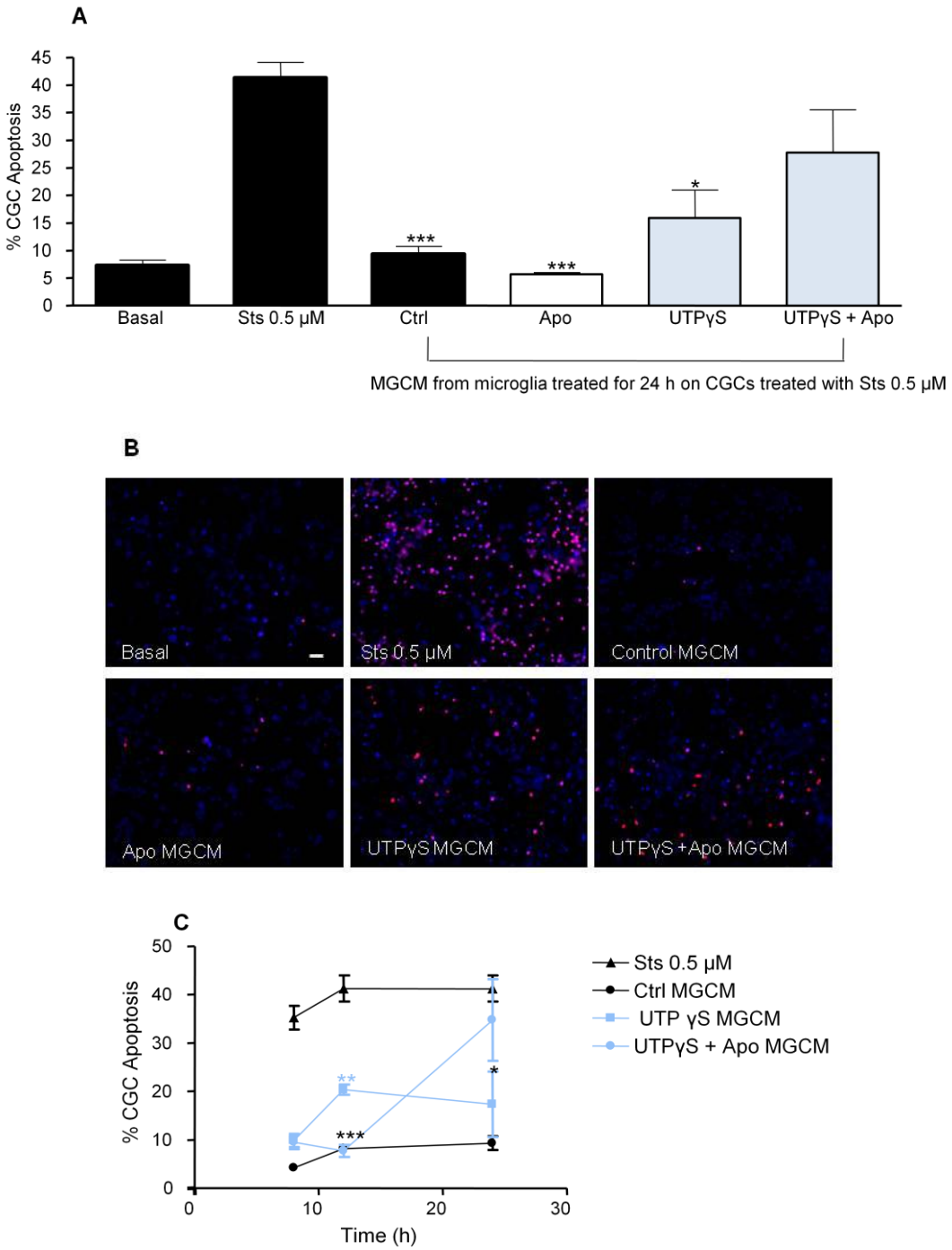

Figure 5.18 Neuronal apoptosis after treatment of CGCs with Sts and MGCM from microglia treated with the P2Y2/4 receptor agonist in the presence or absence of apocynin. *Microglia were either left untreated, treated with apocynin (10 µM) alone or were treated with the P2Y2/4 receptor agonist UTPγS (100 µM) in the presence or absence of apocynin for 24 h before media was taken and added to CGCs treated with Sts (0.5 µM) for 4 h, after which cells were incubated with PI and Hoechst and dead cells were counted to determine the percentage neuronal death* ***(A, B).*** *Microglia were also treated with the P2Y2/4 receptor agonist UTPγS the presence or absence of apocynin for 8 h, 12h, or 24 h* ***(C)****, before media was taken and added to CGCs treated with Sts. CGCs were treated for 4 h and cell death was also assessed by PI and Hoechst 33342 staining to determine the percentage of CGC apoptosis. Data were analysed by one way ANOVA and Tukey post hoc analysis, in which all treatments were compared with Sts treatment alone, and also as indicated. *p<0.05, **p<0.01, ***p<0.001. Data are n=3. Scale bar 20 µm.*

UTPγS MGCM significantly decreased Sts induced neuronal death, whereas UTPγS and apocynin MGCM was not protective against Sts induced apoptosis (Fig. 5.18A), suggesting that UTPγS treatment after 24 h induces a protective effect through superoxide production in an NADPH oxidase dependent manner. The time-course analysis showed that 12 h incubation of microglia with UTPγS plus apocynin significantly decreased CGC death after treatment with Sts (Fig. 5.18C), which also significantly attenuated neuronal death in comparison to treatment with UTPγS MGCM alone. This therefore suggests that at 12 h incubation with UTPγS, microglia release neurotoxic factors in an NADPH oxidase dependent manner, whereas at 24 h incubation with UTPγS, microglia release neuroprotective factors in an NADPH oxidase dependent manner, thereby indicating that the microglial response to P2Y2/4 receptor activation is time dependent.

In summary these data show that NADPH oxidase activation following treatment of microglia with the group I mGluR antagonist MTEP or the group III agonist L-AP4 is neuroprotective through activation of the NADPH oxidase. Activation of the microglial mGluR3 with NAAG is protective; however this effect is not solely dependent on modulation of the microglial NADPH oxidase. Activation of the microglial $GABA_A$ receptor with muscimol is neurotoxic, and this effect could be attenuated by inhibition of the microglial NADPH oxidase with apocynin. Activation of the microglial P2Y2/4 receptor is neroprotective in an NADPH oxoidase manner, as attenuation of P2Y2/4 receptor induced NADPH oxidase activation was neurotoxic. These data therefore show that microglial neurotransmitter receptors modulation mediates neuronal survival in an NADPH oxidase dependent manner.

5.3 Discussion

Treatment of microglia with the neurotransmitters and receptor agonists or antagonists modulated TNFα expression and release in an NADPH oxidase dependent manner, and also affected neuronal survival. The findings here that activation or inhibition of the microglial neurotransmitter receptors modulated NADPH oxidase activity and subsequent neurotoxicity, could provide important information for the production of future therapies for neurodegenerative conditions.

5.3.1 Neurotransmitter mediated microglial TNFα release and ramifications for neuronal survival

Glutamate, GABA or BzATP each induced microglial TNFα expression however, could not enhance TNFα release. These findings could suggest that glutamate, GABA or BzATP may not enhance the expression of the metalloproteinase TNFα cleavage enzyme (TACE), which is responsible for the release of soluble TNFα (Moss & Lambert 2002). In agreement with this suggestion, substance P has been shown to enhance microglial TNFα but not TACE expression, thereby attenuating TNFα release (Zhou et al. 2010). Furthermore, substance P induces NADPH oxidase activation (Block et al. 2006), in the same way that previous reports have demonstrated that BzATP induces NADPH oxidase activity (Parvathenani et al. 2003), and that glutamate, GABA and BzATP can induce NADPH oxidase activation as shown here. It has however been suggested that activation of the Nox1 homologue Duox1 in human epithelial cells with PMA enhances TACE expression and TNFα release, and TACE expression could be attenuated with Rottlerin, a PKCδ and Nox1 inhibitor (Shao & Nadel 2005). This suggests that TACE expression may only be induced by Nox1 mediated superoxide production, and as shown previously, glutmate, GABA and BzATP preferentially activate Nox2 and enhance Nox4 expression, which could account for the enhanced NADPH oxidase mediated TNFα expression but not TNFα release seen here.

Glutamate MGCM enhanced neuronal apoptosis when compared with untreated CGCs. In agreement with the data presented here, exposure of mixed hippocampal neuronal - glial cultures to glutamate for 24 h significantly increased microglial TNFα expression, which was concomitant with increased neuronal TNFR1 expression and apoptosis (Figiel & Dzwonek 2007). Here neuronal apoptosis was further enhanced by glutamate plus apocynin MGCM, suggesting that inhibition of glutamate - induced microglial superoxide production exacerbated the neurotoxic phenotype. In support of this finding, Nox2 activation in a mouse model of MS enhanced acute symptoms, however attenuation of Nox2 promoted the release of inflammatory mediators and enhanced chronic disease severity (Hultqvist et al. 2004), suggesting that attenuation of Nox2 activation is neurotoxic. Neuronal apoptosis following glutamate or glutamate and apocynin MGCM treatment correlated with a significant decrease in Akt phosphorylation (Fig. 5.19A), suggesting that factors released from microglia mediate cell death by attenuating neuronal Akt phosphorylation (Niizuma et al. 2009). In support of this suggestion, cytokines such as TNFα can promote cortical neuron apoptosis through Akt signalling (Rickle et al. 2006).

Glutamate mediates the production of neurotrophic factors through PKC signalling in microglia, which is neuroprotective (Liang et al. 2010). Furthermore, PKC mediates NADPH oxidase activation (Nakajima et al. 2004), and NADPH oxidase derived ROS promote the activation of transcription factors (Pawate et al. 2004), suggesting that glutamate induced NADPH oxidase activation may promote the expression of neurotrophic factors. Here, however, glutamate MGCM enhanced neuronal apoptosis, which supports findings that neurotrophic factors can enhance neuronal apoptosis (Kim et al. 2002). The release of trophic factors from glial cells after damage to the brain can perpetuate neuronal degeneration (Koh et al. 1995; Greenfield & Vaux 2002), suggesting that damaged neurons are susceptible to apoptosis by neurotrophic factors (Greenfield & Vaux 2002). Exposure of cortical neurons *in*

vitro to BDNF induces necrosis (Kim et al. 2002), suggesting that here, microglia may release BDNF after glutamate exposure, which may be enhanced by attenuation of NADPH oxidase activity, suggesting that superoxide production may prevent glutamate induced BDNF release.

Glutamate and thioridazine MGCM reduced glutamate induced neurotoxicity, suggesting that inhibition of glutamate induced Nox4 activation in microglia is neuroprotective. Microglial Nox4 expression enhances IL-6 release (Li et al. 2009) which has neurotoxic consequences in AD, and promotes the cognitive decline seen in animal models of the disease (Lanzrein et al. 1998). In addition, microglial Nox4 activation promotes glutamate release (Harrigan et al. 2008), suggesting that here, glutamate activation of microglial Nox4 may promote neuronal death by glutamate excitotoxicity (Piani & Fontana 1994). Furthermore, microglial NADPH oxidase isoform expression is dependent on the activation state of the cell (Li et al. 2009), and as glutamate can induce a neurotoxic phenotype, particularly through activation mGluR2 (Taylor et al. 2002), it could be suggested that the exposure of microglia to glutamate here may induce microglial reactivity and subsequent Nox4 activation, resulting in the release of neurotoxic factors which may promote neuronal death in a Nox4 dependent manner.

Glutamate MGCM was protective against Sts induced neuronal apoptosis, whilst glutamate plus apocynin MGCM increased Sts induced death. Exposure of microglia to glutamate induces BDNF production, which is protective against excitotoxic neuronal injury (Liang et al. 2010), which could suggest a mechanism whereby glutamate induced NADPH oxidase activation mediates BDNF release that protects neurons against Sts induced apoptosis. Glutamate MGCM from microglia treated with glutamate for 12 h enhanced Sts induced neuronal apoptosis when compared with 24 h treatment. This could suggest a mechanism in which the microglial phenotype changes from neurotoxic to neuroprotective over time, possibly as a consequence of the activation of different Nox isoforms (Li et al. 2009). This

suggestion is supported by findings that 6 h following ischaemia, microglial Nox4 expression is elevated which is followed by enhanced Nox2 expression and activity at later time points in the rat brain (McCann et al. 2008). Although the consequences of this modulation of Nox expression and activity remain to be elucidated, it has been shown that Nox2 and Nox4 activity are temporally regulated in human microglial cells, which may have important consequences for the regulation of the immune response (Li et al. 2009). These findings show that different NADPH oxidase isoforms are activated at different time points following treatment of microglia with glutamate, with differing ramifications for neuronal survival.

GABA MGCM significantly enhanced neuronal death in the presence and absence of Sts, which was attenuated by treatment with GABA plus apocynin MGCM (Fig. 5.19B). Furthermore, GABA significantly elevated microglial TNFα expression in an NADPH oxidase dependent manner. Activation of the microglial GABA recptors mediates cytokine release, with $GABA_B$ receptor activation mediating IL-6 release (Kuhn et al. 2004), which has both pro- and anti-inflammatory properties (Scheller et al. 2011). Furthermore, $GABA_A$ receptor activation in endothelial cells promotes Nox4 activation (Tyagi et al. 2009a), and activation of human microglial Nox4 mediates the constitutive expression and release of IL-6 (Li et al. 2009). Attenuation of microglial Nox4 following GABA treatment enhanced neurotoxicity after application of MGCM to CGCs, indicating that the inhibition of microglial Nox4 following GABA activation may be detrimental to neuronal survival, although the mechanisms involved should be investigated further. In support of these findings, Nox4 activation is protective in a model of retinal cell stress (Groeger et al. 2009); suggesting that GABA induced Nox4 activation may promote microglial derived neuroprotection, whilst activation of microglial Nox1 has been shown to be neurotoxic (Chéret et al. 2008), thereby supporting the findings here that inhibition of microglial GABA induced Nox1/2 activation is neuroprotective.

BzATP enhanced microglial TNFα expression, in accordance with reports that microglial P2X7 receptor activation mediates TNFα production (Suzuki et al. 2004). Furthermore, BzATP MGCM neurotoxicity was attenuated by inhibition of the microglial NADPH oxidase with apocynin (Fig. 5.19B), but not with thioridazine, suggesting that BzATP induced Nox1/2 activation may mediate the release of toxic factors, in line with findings that microglial Nox1 activation is neurotoxic (Chéret et al. 2008). In agreement with the findings here, activation of the microglial P2X7 receptor with BzATP can induce NADPH oxidase activation with neurotoxic consequences (Parvathenani et al. 2003), and ATP treatment of microglia promotes TNFα release (Hide et al. 2000). The enhanced neuronal apoptosis after BzATP MGCM treatment shown here may therefore be a consequence of superoxide induced production of neurotoxic factors. Furthermore, BzATP plus apocynin MGCM attenuated Sts induced neuronal death, suggesting that activation of the microglial NADPH oxidase following BzATP treatment enhances neuronal apoptosis. This was shown to be time dependent, as at 12 h incubation of microglia with BzATP, MGCM was protective against Sts induced neuronal death. These findings concur with suggestions that different microglial NADPH oxidase isoforms are activated at different time points following neuronal damage, which also modulates the expression and release of neurotoxic or neuroprotective factors (McCann et al. 2008). Furthermore, the neurotoxic Nox1 isoform is upregulated 24 h after ischaemia in the rat brain which correlates with neuronal death (Kahles et al. 2010). Acute treatment of microglia with BzATP may therefore induce Nox4 activation, favouring the release of protective factors, whereas chronic treatment may induce Nox1 activation, which mediates the release of toxic factors from microglia.

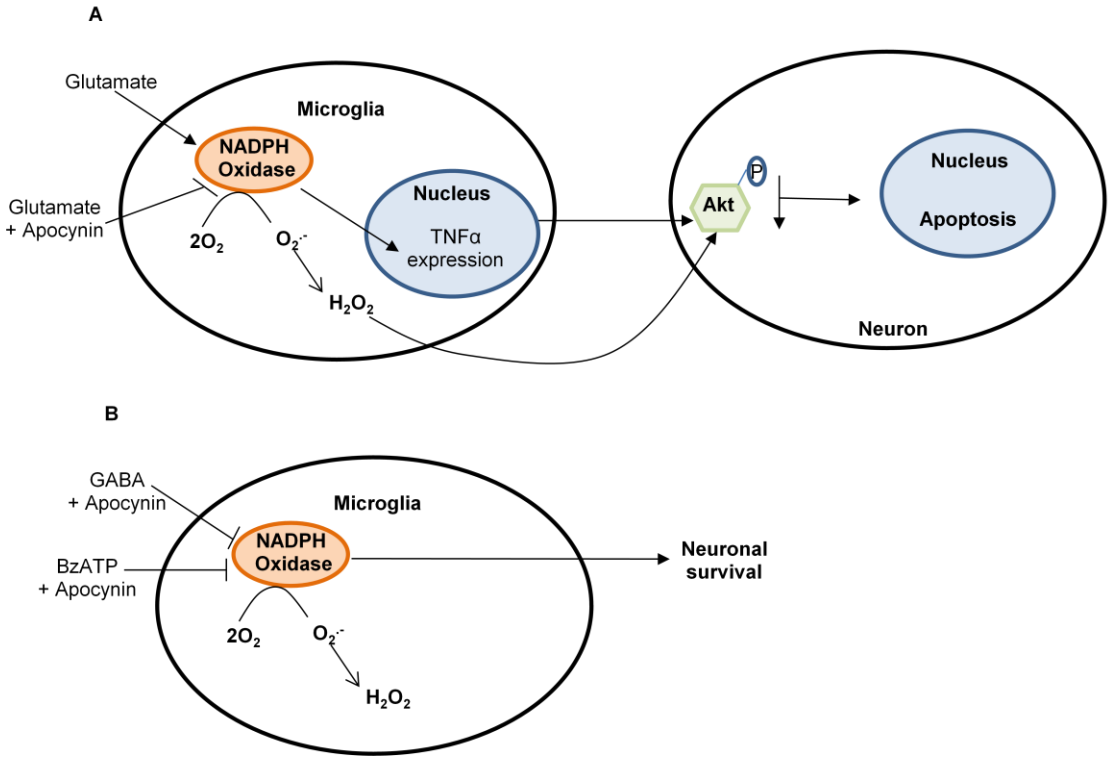

Figure 5.19Schematic of microglial mediated neuronal death or survival following exposure of microglia to neurotransmitters. *Microglia treated with glutamate* ***(A)*** *produce superoxide through activation of the NADPH oxidase, which up regulates TNFα gene expression. Glutamate induced NADPH oxidase activation in microglia reduces Akt phosphorylation which correlates with an increase in neuronal death. Treatment of microglia glutamate plus apocynin attenuates NADPH oxidase activation, however this was concomitant with further decreased Akt phosphorylation and further enhanced neuronal apoptosis. Inhibition of the microglial NADPH oxidase following treatment with GABA or BzATP* ***(B)*** *mediated enhanced neuronal survival, suggesting that GABA or BzATP treatments alone mediate the release of toxic factors in an NADPH oxidase dependent manner.*

5.3.2 Receptor mediated microglial TNFα production and ramifications for neuronal survival

Microglial mGluR modulation affected neuronal survival through NADPH oxidase activation. Treatment of microglia with the group I mGluR antagonist MTEP did not elevate TNFα expression or release; however, protein levels were significantly enhanced in an NADPH oxidase dependent manner. The discrepancy between gene and protein expression could suggest a role for MTEP induced NADPH oxidase activation in the regulation of translation. Nox2 and Nox4 co-localise with the ER marker calreticulin in endothelial cells,

suggesting a role in the regulation of protein folding and translation (Petry et al. 2006). Furthermore, in human smooth muscle cells, Nox4 activation promotes the phosphorylation and activation of the eukaryotic translation initiation factor 4E binding protein-1 (4E- BP1), which regulates protein synthesis (Sturrock et al. 2007). Antagonism of the group I mGluRs may therefore induce TNFα translation through enhanced NADPH oxidase activity, rather than induction of transcription.

The elevated microglial TNFα protein expression after inhibition of the group I mGluR correlates with findings that inhibition of the microglial group I mGluR in rat spinal cord injury promotes microglial activation and cytokine release (Byrnes et al. 2009). Here however, MTEP treatment of microglia did not promote TNFα release. The differences reported here and by Byrnes et al (2009) may be a result of the *in vivo* nature of the published report. Neuronal damage provides additional microglial activating stimuli, such as ATP that is released from damaged neurons (Franke et al. 2006) and acts on microglial P2X7 receptors to promote TNFα release from microglia (Suzuki et al. 2004); suggesting that antagonism of the microglial group I mGluRs may contribute to, rather than induce, microglial reacitivity. The findings here therefore indcicate that antagonism of the microglial group I mGluRs enhances TNFα protein expression in an NADPH oxidase dependent manner in the absence of other stimuli; however additional activating stimuli may be required to induce TNFα release (Loane et al. 2009) (Fig. 5.20).

MTEP MGCM did not significantly enhance neuronal apoptosis, however further protection was observed following treatment with MTEP plus apocynin MGCM, although because this protection was not significantly more than that seen with control MGCM, it could be suggested that this is not a consequence of attenuation of the NADPH oxidase. The findings however that both activation and inhibition of MTEP induced NADPH oxidase activation are

protective could suggest that inhibition of the microglial group I mGluRs also affects neuronal survival through an NADPH oxidase independant pathway. In support of this suggestion, attenuation of the microglial group I mGluRs mediates neuroprotection after ischaemia *in vivo* (Kohara et al. 2008); however the mechanisms behind this protection are not well understood (Byrnes et al. 2009).

Neuronal Akt phosphorylation was enhanced upon treatment of CGCs with MTEP MGCM, correlating with neuronal survival (Fig. 5.20). Furthermore, neuronal caspase 12 cleavage was not affected by MTEP or MTEP plus apocynin MGCM, lending further support to the suggestion that NADPH oxidase activation after inhibition of microglial group I mGluRs may be protective. Neuronal TNFR1 expression was significantly enhanced following treatment with MTEP or MTEP plus apocynin MGCM, which could suggest a mechanism of neuroprotection through enhanced TNFR1 shedding (Bartsch et al. 2010), thereby preventing the binding of TNFα to membrane bound receptors, attenuating apoptosis (Pinckard et al. 1997).

MTEP MGCM was neuroprotective against Sts induced apoptosis, which was reversed by attenuation of NADPH oxidase activation, suggesting that inhibition of the microglial NADPH oxidase enhanced the death of damaged neurons. As previous studies have reported that activation of the microglial mGluR group I induces a neuroprotective phenotype through inhibition of the NADPH oxidase (Loane et al. 2009, Byrnes et al. 2009) it could be suggested that inhibition of the group I mGluR is protective through activation of protective isoforms of the NADPH oxidase, such as Nox2 (Chéret et al. 2008; Groeger et al. 2009) and that inhibition of this superoxide production induces the release of neurotoxic substances.

Treatment of microglia with the mGluR3 agonist NAAG significantly elevated TNFα protein expression, but not release, in an NADPH oxidase dependent manner (Fig. 5.20). This

NADPH oxidase induced TNFα production did not enhance neuronal apoptosis (Fig. 5.20); however NAAG plus apocynin MGCM offered significant neuroprotection. Furthermore, both NAAG and NAAG plus apocynin MGCM were protective against Sts induced neuronal apoptosis. This could suggest that activation of the microglial mGluR3 is protective in both an NADPH oxidase dependent and independent manner. In support of this suggestion, it has been shown that NAAG MGCM (from microglia treated for 24 h) is not neurotoxic (Taylor et al. 2005). Furthermore, activation of microglial mGluR3 is protective in a variety of diseases. Glial mGluR3 expression is upregulated in a rat model of temporal lobe epilepsy, and is responsible for neuroprotection through the release of TGFβ (Aronica et al. 2000). Furthermore, activation of glial mGluR3 promotes IL-6 release, which has plieotroic properties (Scheller et al. 2011), has been shown to be protective in rat models of stroke (Loddick et al. 1998), and can promote neuronal survival *in vitro* (März et al. 1997). It could therefore be suggested that activation of the microglial mGluR3 induces the production of protective cytokines, which is not solely dependent on activation of the NADPH oxidase.

This cytokine production could however, be partially dependent on NADPH oxidase activation, as neuroprotection was enhanced upon attenuation of mGluR3 induced NADPH oxidase activation. As mentioned previously, many cytokines have pleiotropic properties (Scheller et al. 2011), and it could be suggested that the activation of the NADPH oxidase up-regulates the production of these cytokines, however, attenuation of their production may confer further protection, as shown here. For example, IL-6 is up-regulated by Nox4 activation in a human microglial cell line, which modulates the inflammatory response in neurodegenerative disease (Li et al. 2009). Whilst IL-6 release may be neuroprotetcive (März et al. 1997), the inhibition of NADPH oxidase induced IL-6 production further enhances neuroprotection (Turchan-Cholewo et al. 2009), thereby suggesting that if NAAG mediates the expression and release of plieotropic cytokines in an NADPH oxidase dependent manner,

neuroprotection may be further enhanced by inhibition of NAAG induced NADPH oxidase activation. Together these data support a role for microglial mGluR3 activation in neuronal survival through modulation of NADPH oxidase activity, however the data and evidence from published findings could suggest that attenuation of mGluR3 induced NADPH oxidase activation may further enhance neuronal survival.

Further investigations into neuronal apoptosis showed that NAAG MGCM did not modulate neuronal caspase 12 cleavage, however, in line with the study of neuronal death, neuronal caspase 12 cleavage was significantly attenuated after NAAG and apocynin MGCM treatment. Neuronal TNFR1 expression was significantly enhanced upon treatment with NAAG MGCM, which was down-regulated by treatment with NAAG plus apocynin. This could again suggest a protective mechanism in which the shedding of TNFR1 prevents death signalling from the binding of TNFα to membrane bound receptors (Bartsch et al. 2010). Furthermore, expression of microglial TNFα protien may promote the up-regulation of TNFR1 expression, which has been shown in slice cultures (Harry et al. 2008), and here both TNFα and TNFR1 protien levels were attenuated by inhibition of the mGluR3 NADPH oxidase activation. These findings therefore suggest that mGluR3 NADPH oxidase activation is implicated in neuroprotection, however other pathways may also be involved.

Activation of the microglial group III mGluR with L-AP4 did not modulate TNFα protein expression or release, however, treatment of microglia with L-AP4 and apocynin significantly elevated TNFα gene expression. Furthermore, L-AP4 plus apocynin MGCM significantly elevated neuronal death, which also correlated with increased neuronal caspase 12 cleavage and TNFR1 expression, suggesting that neuronal death following the inhibition of microglial group III mGluR mediated NADPH oxidase activation was a consequence of the activation of these death cascades (Fig. 5.20), and that apoptosis was mediated by the

induction of ER stress (Shibata & Kobayashi 2008) as a consequence of L-AP4 plus apocynin MGCM treatment.

Activation of the microglial group III mGluR with L-AP4 is neuroprotective (Taylor et al. 2003), and can protect against myelin induced neurotoxicity (Pinteaux-Jones et al. 2008). In addition, treatment of mixed neuronal – glial cultures with L-AP4 is protective against Aβ induced neuronal apoptosis (Copani et al. 1995), and activation of group III mGluRs with L-AP4 *in vivo* is protective against 6-hydroxydopamine toxicity in a model of PD (Vernon et al. 2007). In support of these published findings, L-AP4 MGCM was protective against Sts induced neurotoxicity, which was attenuated upon treatment of Sts treated CGCs with L-AP4 plus apocynin MGCM, suggesting that activation of the microglial group III mGluRs is protective through activation of the NADPH oxidase.

The finding here that attenuation of microglial group III mGluR mediated superoxide production promotes neurotoxicity suggests that activation of group III mGluRs mediates protection in a Nox2 dependent manner. Nox2 activation is protective in models of autoimmune disease (Hultqvist et al. 2009), and has been shown to decrease disease severity in a mouse model of MS (Hultqvist et al. 2004). Furthermore, Nox2 activation aids recovery after ischaemia in *in vivo* rat models, in which Nox2 knockout mice showed larger infarct volumes than wild type mice after ischaemia (Kunz et al. 2007), and Nox2 activation provides neuroprotection against excitotoxic injury *in vivo* (Kawano et al. 2007). This, and the data presented here therefore suggest that microglial group III mGluR activation of Nox2 is protective.

Preliminary investigations showed that modulation of the microglial iGluRs affected neuronal survival, however, controls whereby CGCs were directly treated with the iGluR modulators were not conducted, and would be needed to ensure that the effects seen were not a

consequence of direct modulation of neuronal iGluRs. However, NMDA MGCM did not increase neuronal apoptosis, whereas NMDA plus apocynin MGCM significantly elevated neuronal death. Treatment of microglia with glutamate at levels consistent with activation of the NMDA receptor mediates the release of protective factors (Liang et al. 2010), suggesting that the neuroprotection seen here may be due to NMDA receptor mediated release of neurotrophic factors in an NADPH oxidase dependent manner. Microglial activation of the NMDA receptor *in vivo* induces the translocation of NF-κB to the nucleus (Murugan et al. 2011), which promotes the transcription of cytokines such as TNFα in a ROS dependent manner (Quan et al. 2011), which under certain conditions may be protective (Kraft et al. 2009). Furthermore, NADPH oxidase activation mediates the activity of transcription factors (Pawate et al. 2004); therefore NMDA receptor induced ROS production may mediate cytokine expression. TNFα is protective against neuronal excitotoxicity (Marchetti et al. 2004), and it could therefore be suggested that NMDA mediated release of TNFα could promote the neuroprotection seen here.

Activation of microglia with NMDA increased TNFα expression, which was further enhanced by co-treatment with NMDA and apocynin, which also correlated with an increase in neuronal death. It has been suggested that low levels of NMDA receptor activation and subsequent TNFα release enhances neuronal survival before ischaemia, and that this pre-conditioning may be protective (Watters & O'Connor 2011). The low levels of TNFα released from microglia treated with NMDA may protect neurons from apoptosis, as seen here; whereas elevated levels of TNFα induced by inhibition of NMDA induced NADPH oxidase activation may promote neuronal apoptosis. The findings presented here therefore suggest that inhibition of microglial NMDA induced NADPH oxidase activation may be neurotoxic through the production of TNFα which facilitates neuronal death. Microglial NMDA receptor mediated NADPH oxidase activation may therefore be protective.

Inhibition of the microglial AMPA receptor with CNQX enhanced TNFα expression and elevated neuronal death in an NADPH oxidase dependent manner, which supports published findings that activation of the AMPA receptor does not induce Nox4 activation and H_2O_2 production in cortical neurons, which is neuroprotective (Ha et al. 2010). Suggesting that antagonism of the microglial AMPA receptor may mediate TNFα expression in a Nox4 dependent manner, in agreement with findings that Nox4 can up-regulate IL-6 expression (Li et al. 2009). Previous studies have shown that microglial AMPA receptor activation can induce TNFα release (Noda et al. 2000), and co-treatment of microglia with the AMPA receptor agonist and the antagonist CNQX can attenuate microglial TNFα release (Hagino et al. 2004). It is not known whether inhibition of the AMPA receptor alone modulates the expression or release of TNFα, however, the data presented here show that TNFα expression is elevated following CNQX treatment in an NADPH oxidase dependent manner, which is detrimental to neuronal survival.

Activation of the microglial $GABA_A$ receptor significantly increased TNFα release in an NADPH oxidase dependent manner, which correlated with increased neuronal apoptosis that could be attenuated by co-treatment of microglia with muscimol and apocynin MGCM (Fig. 5.20). Muscimol plus apocynin MGCM also attenuated Sts induced neuronal apoptosis, suggesting that inhibition of microglial $GABA_A$ receptor induced superoxide production is protective to damaged neurons.

Neuronal caspase 12 cleavage was attenuated by co-treatment of microglia with muscimol and apocynin. Modulation of the microglial $GABA_A$ receptor therefore induces TNFα production in an NADPH oxidase dependent manner, which induces neuronal apoptosis through caspase 12 activation and an ER stress pathway. This apoptotic pathway is seen in cardiomyocytes subjected to ischemic conditions, whereby TNFα is elevated and activates apoptosis through caspase 12 cleavage (Bajaj & Sharma 2006), and has also been shown in

fibroblasts *in vitro*, whereby TNFα application can mediate caspase 12 dependent apoptosis (Kalai et al. 2003). Neuronal TNFR1 expression was not modulated by muscimol MGCM, however TNFR1 expression was increased following treatment of CGCs with muscimol plus apocynin MGCM. This may represent a protective mechanism in which TNFR1 is shed from neurons to prevent TNFα binding to TNFR1 on the membrane and subsequent neuronal apoptosis, which has been demonstrated in a mouse model of motor neurone disease (Bartsch et al. 2010), which also agrees with the protective properties of muscimol plus apocynin MGCM seen here. CGCs treated with muscimol plus apocynin MGCM may therefore elevate the production of soluble TNFR1 to facilitate neuroprotection. The low level of TNFR1 expression following exposure of neurons to muscimol MGCM could suggest that the receptor has been internalised and degraded following binding to TNFα (Harry et al. 2008), which correlates with enhanced neuronal death. Interestingly, Akt phosphorylation was not modulated by treatment of neurons with muscimol MGCM, however, it has been suggested that TNFα can mediate Akt phosphorylation in fibroblasts (Chen et al. 2006), which was seen here.

The $GABA_A$ receptor is up-regulated on activated glia, in particular in gliomas (Synowitz et al. 2001), suggesting that $GABA_A$ receptor expression is enhanced on activated microglia, suggesting a neurotoxic role. Furthermore, application of GABA to lymphocytes can induce cytotoxcity (Bergeret et al. 1998), supporting the findings here that $GABA_A$ receptor activation may induce the release of toxic factors from microglia. Furthermore, application of muscimol to adult rat brain slices induced an increase in the release of the inflammatory chemokine MIP-1α (Cheung et al. 2009), which is up-regulated in the MPTP model of PD and contributes to neuronal apoptosis (Kalkonde et al. 2007). In addition, $GABA_A$ receptor activation in endothelial cells promotes Nox4 activation and ERK modulation (Tyagi et al. 2009), which, in microglia, is consistent with an activated phenotype and the production of

pro-inflammatory cytokines (Qian et al. 2008). Taken together, these published data support a role for microglial $GABA_A$ receptor activation in the release of neurotoxic factors in an NADPH oxidase dependent manner, resulting in neuronal death.

Activation of the microglial P2Y2/4 receptor significantly elevated TNFα protein expression, however this was not modulated by co-treatment with apocynin, although microglial TNFα release was enhanced by treatment with UTPγS in an NADPH oxidase dependent manner (Fig. 5.20). Neuronal apoptosis was not significantly elevated by UTPγS MGCM, however further neuroprotection was seen following treatment with UTPγS plus apocynin MGCM (Fig. 5.20), suggesting that attenuation of TNFα release as a consequence of UTPγS mediated NADPH oxidase inhibition is protective. Neuronal caspase 12 clevage was not significantly altered following treatment with UTPγS MGCM, however neuronal TNFR1 expression was significantly enhanced in the presence of UTPγS MGCM or UTPγS plus apocynin MGCM, suggesting that the presence of TNFα may promote TNFR1 shedding from neurons to facilitate protection (Bartsch et al. 2010).

UTPγS MGCM mediated neuronal apoptosis was not significant, however, attenuation of microglial P2Y2/4 receptor induced NADPH oxidase activation significantly enhanced neuroprotection, suggesting a slight toxic effect of P2Y2/4 receptor mediated microglial NADPH oxidase activation. Activation of microglial P2Y2/4 receptors can attenuate microglial reactivity after exposure to LPS (Boucsein et al. 2003); however, TNFα release from microglia has been observed following treatment with UTP (Ogata et al. 2003). Activation of the microglial P2Y receptors is implicated in the recruitment of leukocytes to the brain tissue, which could have important ramifications for inflammatory processes (Boucsein et al. 2003). Furthermore, Nox2 activation in macrophages can regulate the immune response by activating T-cells (Hildeman et al. 2003), and it could therefore be suggested that activation of microglial P2Y2/4 receptors, which mediates microglial

chemotaxis (Inoue 2002), may also promote the activation of Nox2 which may facilitate the recruitment of immune cells to the sites of neuronal injury, in addition to having a slight toxic effect through the release of cytokines, which may be a consequence of Nox1 activation (Chéret et al. 2008). In support of this suggestion, activation of the microglial P2Y receptors was found to enhance PMA induced superoxide production which elevated TNFα release (Ogata et al. 2003). Attenuation of this NADPH oxidase activation may therefore promote the neuroprotective effects seen here following treatment of CGCs with UTPγS plus apocynin MGCM.

Neuronal apoptosis following treatment with Sts was attenuated by treatment with UTPγS MGCM. In support of these findings, activation of the microglial P2Y receptors with UTP is neuroprotective against hypoxic insult through the release of TNFα (Morigiwa et al. 2000). This therefore suggests that TNFα release following UTPγS mediated NADPH oxidase activation may be protective to apoptotic neurons. Furthermore, Akt phosphorylation remained elevated following treatment of neurons with UTPγS MGCM, suggesting that TNFα release may confer protection through Akt phosphorylation, as shown in fibroblasts (Chen et al. 2006). This is supported by findings from Groeger et al. (2009), who show that Nox2 and Nox4 activation is protective against retinal cell stress induced *in vitro*. Purinergic receptor mediated NADPH oxidase activation can enhance cytokine release (Brown & Griendling 2009) which can feedback onto microglia, inducing further Nox4 activation (Basuroy et al. 2009), which mediates protection (Groeger et al. 2008). This could account for the attenuation of apoptosis of Sts treated neurons following application of UTPγS MGCM. Activation of the microglial P2Y2/4 receptor and subsequent NADPH oxidase activation therefore promotes TNFα production, which may be both protective and toxic.

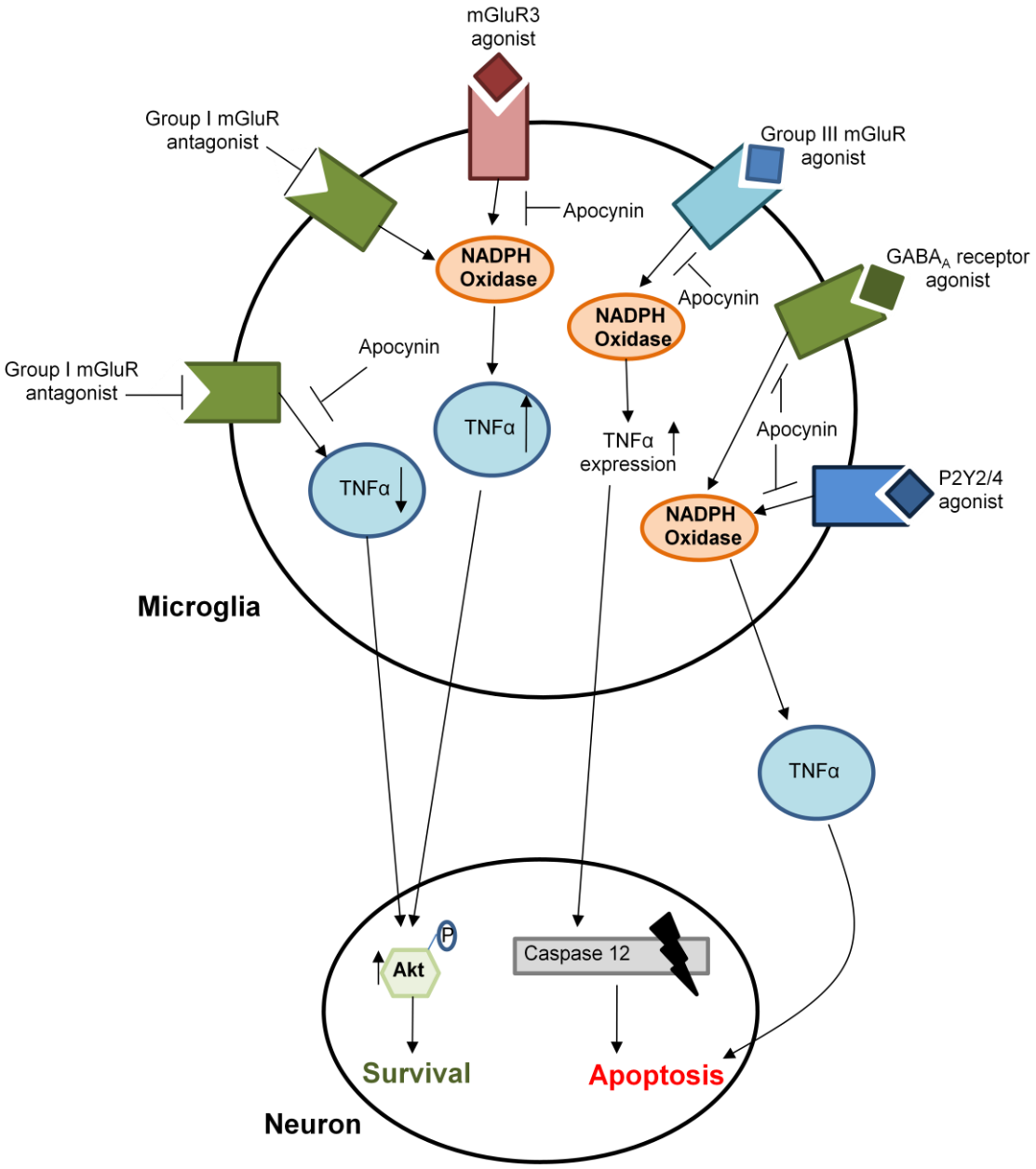

Figure 5.20 Summary of the mechanisms of neuronal survival and death after modulation of the microglial NADPH oxidase following treatment with receptor agonists or antagonists. *Treatment of microglia with the mGluR group I antagonist MTEP induced a significant increase in TNFα protein expression in an NADPH oxidase dependent manner, which mediated neuronal Akt phosphorylation and survival. Inhibition of mGluR group I mediated NADPH oxidase activation decreased TNFα expression, which further enhanced neuronal survival. Treatment of microglia with the mGluR3 agonist NAAG increased TNFα expression which mediated Akt phosphorylation and neuronal survival, however inhibition of mGluR3 induced superoxide production also favoured neuronal survival through the inhibition of TNFα expression. Attenuation of mGluR group III mediated superoxide production enhanced TNFα gene expression which correlated with an increase in neuronal caspase 12 clevage and apoptosis, suggesting activation of the microglial mGluR group III is protective through modulation of the NADPH oxidase. Treatment of microglia with the GABA$_A$ or P2Y2/4 receptor agonists induced an increase in TNFα release which promoted increased neuronal apoptosis when compared with treatments in the presence of apocynin, which could also attenuate TNFα release and neuronal apoptosis.*

5.3.3 Conclusions

The data presented here suggest that modulation of the microglial neurotransmitter receptors mediate intracellular superoxide production that is used as a signalling molecule to promote the production of neuroprotective or neurotoxic factors, which has ramifications for neuronal survival. Attenuation of the microglial group I mGluRs mediates Nox1 and Nox2 derived superoxide production, which promotes the production and release of neuroprotective factors and favours neuronal survival. Activation of the microglial mGluR3 or the group III mGluRs promotes superoxide production in a Nox2 and Nox4 dependent manner, which also has neuroprotective properties. Activation of the $GABA_A$ receptor and the P2Y2/4 receptors facilitated superoxide production in a Nox1 dependent manner, which promoted neurotoxicity through the production and release of TNFα. Furthermore, neuronal apoptosis induced by microglial conditioned media from microglia treated with muscimol induced caspase 12 cleavage, suggesting an ER stress drived mechanism of neuronal death, which could be attenuated by inhibition of the microglial NADPH oxidase. These findings therefore suggest that modulation of the microglial mGluRs mediates neuronal survival in an NADPH oxidase dependent manner, whilst activation of the $GABA_A$ or P2Y2/4 receptors may promote neurotoxicity in an NADPH oxidase dependent manner. The modulation of microglial neurotransmitter receptors may therefore have important ramifications for the regulation of the inflammatory response through mediating NADPH oxidase activation, which could have imortant consequences for the treatment of neuroinflammatory conditions.

Chapter 6

General Discussion

6. General Discussion

The research presented in this thesis focused on investigating the mechanisms behind, and subsequent ramifications of microglial NADPH oxidase activation in response to neurotransmitters and modulation of microglial neurotransmitter receptors. The data presented show that microglial neurotransmitter receptor mediated NADPH oxidase activation can regulate neuronal survival, which provides important information for the generation of future therapies for neurodegenerative conditions.

Neurotransmitter dysregulation, microglial reactivity, and ROS production are a hallmark of several neurodegenerative diseases. Excitatory neurotransmitters such as glutamate are present in the CSF of MS patients (Stover et al. 1997), and GABA is released into the extracellular space during ischaemia, which may occur in an attempt to counteract the excitatory actions of glutamate, to promote neuroprotection (Hutchinson et al. 2002). Glutamate is released from astrocytes and neurons during neurodegenerative conditions, which contributes to neuronal excitotoxicity and microglial activation (Rossi & Volterra 2009). Furthermore, in AD, GABAergic neurons in the hippocampus are not susceptible to death, and may contribute to the abnormal neuronal activity through the release of GABA, thereby perpetuating disease progression (Davies et al. 1998). Furthermore, the GABA tone in post-mortem AD brains is elevated in comparison to controls, suggesting that GABAergic transmission may contribute to AD pathology (Marczynski 1998). In addition, GABA accelerates excitotoxic death in cortical neuronal cultures through activation of $GABA_A$ receptors (Erdö et al. 1991). ATP levels are also elevated after neuronal injury (Volonté et al. 2003), which can act on microglia to induce chemotaxis to the site of neuronal damage (Nimmerjahn et al. 2005). Pathological levels of ATP (1 mM) promote microglial activation and subsequent NADPH oxidase dependent ROS production (Parvathenani et al. 2003), in

addition to the release of cytokines such as TNFα (Hide et al. 2000) with detrimental consequences.

In addition to the pathological role of neurotransmitter dysregulation, microglial NADPH oxidase activation is considered detrimental to neuronal survival. The microglial NADPH oxidase is activated by Aβ and contributes to AD progression (Wilkinson & Landreth 2006; Block 2008), and microglial NADPH oxidase activation is also seen in PD, and is induced by toxins such as paraquat (Miller et al. 2007), MPTP and rotenone (Gao et al. 2003). Furthermore, microglial NADPH oxidase activation is seen in MS, in which ROS production is induced by myelin phagocytosis, which contributes to neurotoxicity (Liu et al. 2006). Attenuation of microglial NADPH oxidase activity following activation by Aβ (Wilkinson et al. 2010), rotenone (Gao et al. 2003), paraquat (Miller et al. 2007), or myelin (Liu et al. 2006), can protect against neurotoxicity.

Microglia express a wide repertoire of neurotransmitter receptors (Pocock & Kettenmann 2007), which respond to physiological and pathological levels of neurotransmitters with varying ramifications for microglial reactivity and disease progression. Furthermore, microglia also express three isoforms of the NADPH oxidase: Nox1 (Chéret et al. 2008), Nox2 (Wilkinson & Landreth 2006) and Nox4 (Harrigan et al. 2008). It was therefore considered important to investigate the hypothesis that modulation of microglial neurotransmitter receptors may induce NADPH oxidase activation which may promote either a neurotoxic or neuroprotective phenotype.

6.1 Modulation of microglial neurotransmitters induces NADPH oxidase activation with ramifications for neuronal survival

The data presented here demonstrated that exposure of microglia to glutamate, GABA or ATP induced NADPH oxidase activation. Whilst exposure of microglia to ATP and

activation of P2X7 receptors has been shown to mediate NADPH oxidase activation (Parvathenani et al. 2003), it has not been previously reported that exposure of microglia to glutamate or GABA can mediate NADPH oxidase activity. Following on from these studies, the role of neurotransmitter receptors in superoxide production was investigated. Inhibition of the microglial group I mGluRs, activation of the group II mGluR3, activation of the group III mGluRs, modulation of the microglial iGluRs, and activation of the $GABA_A$ and P2Y2/4 receptors were shown to induce superoxide production through activation of different NADPH oxidase isoforms, with consequences for neuronal survival.

Superoxide production following modulation of the microglial glutamate receptors was neuroprotective through activation of NADPH oxidase isoforms (Fig. 6.1, Table 8). Inhibition of the microglial group I mGluRs with MTEP induced Nox1 and Nox2 mediated superoxide production through PKC and PI3-K activation and p44/42ERK induction Furthermore, MTEP induced superoxide production from Nox1 and Nox2 activation mediated p44/42ERK phosphorylation (Fig. 6.1). MTEP induced superoxide production also elevated TNFα expression, and application of MTEP MGCM to CGCs was protective through enhanced Akt phosphorylation (Fig. 6.1).

Treatment of microglia with the mGluR group I agonist can down-regulate NADPH oxidase activation, which is protective in models of spinal trauma (Byrnes et al. 2009) and *in vitro* (Loane et al. 2009). This supports findings here that antagonism of the group I mGluRs promotes superoxide production, however, as inhibition of the microglial group I mGluR did not induce neuronal apoptosis, MTEP induced superoxide production may be mediated by a Nox isoform considered to be protective. Nox2 activation can protect against immune mediated degeneration in a mouse model of rheumatoid arthritis (Hultqvist et al. 2009), and attenuates disease severity in a mouse model of MS (Hultqvist et al. 2004). Furthermore, Nox2 activation is protective in models of ischaemia (Kunz et al. 2007) and excitotoxicity

(Kawano et al. 2007). The data presented here therefore suggests that attenuation of the microglial group I mGluR promotes neuroprotection through Nox2 activation.

Interestingly, treatment of microglia with MTEP plus apocynin up-regulated Nox1 expression, which also correlated with increased neuronal death following treatment with the apoptotic inducer staurosporine and MTEP plus apocynin MGCM, however healthy neurons were not affected by this treatment. Activation of the microglial Nox1 is neurotoxic, mediating the release of IL-1β, and promoting neuronal death *in vitro* (Chéret et al. 2008), and attenuation of Nox1 activation following ischaemia in the rat brain promotes neuroprotection (Kahles et al. 2010). IL-1β has no effect on the survival of healthy neurons *in vitro,* only enhancing degeneration after excitotoxic injury (Stone & Behan 2007), lending support to the suggestion that Nox1 induction after MTEP plus apocynin treatment may mediate IL-1β production to enhance the apoptosis of staurosporine damaged neurons only. Attenuation of MTEP induced Nox2 activation may mediate the expression and activation of Nox1 through MTEP induced PKC signalling, which has been shown to up-regulate Nox1 expression (Wei et al. 2010) and activity (Fan et al. 2005). Furthermore, a neurotoxic NADPH oxidase is preferentially induced after treatment of microglia with paraquat, which also mediates PKC and p44/42ERK signalling (Miller et al. 2007), lending support to the findings here that antagonism of the microglial group I mGluR may mediate Nox1 activation through PKC and p44/42ERK activation. The data presented here therefore show that Nox2 activation by group I mGluR antagonism represents a neuroprotective phenotype, whilst activation of Nox1 following Nox2 inhibition enhances neuronal death.

Activation of the microglial mGluR3 induced superoxide production through Nox2 and Nox4 activation (Fig. 6.1, Table 8), and induced a neuroprotective phenotype, in line with findings from Taylor et al. (2005), who demonstrated that mGluR3 activation with NAAG could not induce microglial reactivity or neurotoxicity after treatment with peptides associtated with

AD; and correlating with findings that activation of mGluR3 reduces microglial reactivity in response to myelin exposure (Pinteaux-Jones et al. 2008). Furthermore, Nox2 and Nox4 activation is protective in a model of retinal cell stress (Groeger et al. 2009); supporting the findings here that microglial mGluR3 activation is protective through Nox2 and Nox4.

Activation of microglial Nox2 following NAAG treatment may mediate the protective phenotype. Attenuation of NAAG induced Nox2 activation with apocynin could not mediate the same level of neuroprotection in staurosporine treated neurons. This could suggest that Nox4 activation (which is not inhibited by apocynin) may induce the release of cytokines such as IL-6 (Li et al. 2009), which may not protect apoptotic neurons in the same ways that IL-6 provides protection to healthy neurons (Scheller et al. 2011). In support of this suggestion, activation of the astrocytic mGluR3 induces IL-6 release (Aronica et al. 2005), which could be through a Nox4 dependent mechanism (Li et al. 2009). These studies therefore lend support to the findings here that activation of the microglial mGluR3 mediates neuronal protection through Nox2 activation, and that Nox2 inhibition may enhance Nox4 activity, thereby slightly attenuating the survival of staurosporine treated neurons.

In this thesis, activation of the microglial mGluR3 mediated superoxide production in a p38MAPK and p44/42ERK dependent manner; and superoxide production through Nox2 and Nox4 activation also induced phopshorylation of these signalling molecules (Fig. 6.1). In astrocytes, mGluR3 activation mediates p38MAPK and p44/42ERK activation which facilitates survival (Ciccarelli et al. 2007). It could therefore be suggested that this MAPK activation is perpetuated by NADPH oxidase induction after mGluR3 activation, thereby promoting the protective phenotype seen here and reported by Taylor et al (2005). Microglial mGluR3 activation also induced TNFα expression, which mediated neuronal survival in an NADPH oxidase dependent manner (Fig. 6.1). The protective effects of mGluR3 activation are therefore mediated by Nox2 activity, which facilitates TNFα production with protective

consequences for healthy neurons, mediating homeostasis and protection against insults (Sriram & O'Callaghan 2007). Furthermore, TNFα mediates the survival of fibroblasts in an Akt dependent manner (Chen et al. 2006), which correlates with findings here that neuronal Akt phosphorylation was up-regulated following exposure to NAAG MGCM. These findings therefore also support a role for microglial Nox2 activation in protection, and could represent a mechanism whereby microglial Nox2 activation through treatment with mGluR3 agonists could regulate the immune response in neurodegeneration, as seen in other immune diseases (Hultqvist et al. 2009).

Activation of the microglial group III mGluR mediated neuroprotection through NADPH oxidase activation. Treatment of microglia with the group III agonist L-AP4 induced p38MAPK activation which mediated Nox2 and Nox4 activation, down-regulating TNFα expression and promoting neuronal survival (Fig. 6.1, Table 8). Furthermore, attenuation of the microglial NADPH oxidase after activation of the group III mGluR significantly increased neuronal apoptosis, which correlated with increased TNFα expression. Activation of microglial group III mGluRs mediates protection against exposure of microglia to myelin (Pinteaux-Jones et al. 2008), and down-regulates microglial reactivity in response to CgA or Aβ, with neuroprotective consequences (Taylor et al. 2003). Furthermore, activation of microglia with glutamate at levels concurrent with group III mGluR activation mediates the production of neurotrophic factors in a PKC dependent manner (Liang et al. 2010), and as NADPH oxidase activation is induced by PKC activity (Yamamori et al. 2000), it could be suggested that activation of the microglial group III mGluRs mediates neuroprotection through the production of neurotrophic factors in an NADPH oxidase dependent manner. In addition, activation of group III mGluRs expressed on a stably transfected cell line induces p38MAPK activity (Ferraguti et al. 1999), which promotes a protective phenotype in astrocytes (Ciccarelli et al. 2007). Together these published findings and the data presented

here show that activation of the microglial group III mGluRs mediates neuronal survival through the release of trophic factors in a Nox2 dependent manner through the p38MAPK signalling cascade.

Treatment of microglia with the AMPA receptor agonists did not mediate superoxide production, and did not significantly modulate neuronal survival, although attenuation of the AMPA receptor enhanced neuronal death in an NADPH oxidase dependent manner. However, it has been shown that activation of the AMPA receptor has little effect on neuronal Nox4 activation in comparison with activation of the NMDA receptor, which had a greater effect on Nox4 activity (Ha et al. 2010), in line with the data presented here. Activation of the microglial NMDA receptor mediated superoxide production in an NADPH oxidase dependent manner through a p38MAPK dependent mechanism (Fig. 6.1, Table 8). Furthermore, activation of the microglial NMDA receptor down-regulated TNFα expression in an NADPH oxidase dependent manner, and also mediated neuronal survival (Fig. 6.1). Microglial NMDA receptor activation can induce the production of neurotrophic cytokines in a PKC dependent manner (Liang et al. 2010), and PKC also mediates NADPH oxidase activation (Fan et al. 2005), suggesting that here, microglial NMDA receptor activation could mediate neuronal survival in an NADPH oxidase dependent manner. In addition, activation of the microglial group II mGluRs can induce NMDA receptor activation through a PKC dependent pathway in cortical neurons (Tyszkiewicz et al. 2004), and it could therefore be suggested that in microglia, activation of the group II mGluRs and subsequent NMDA receptor activation could perpetuate superoxide production through activation of the NADPH oxidase, with neuroprotective consequences.

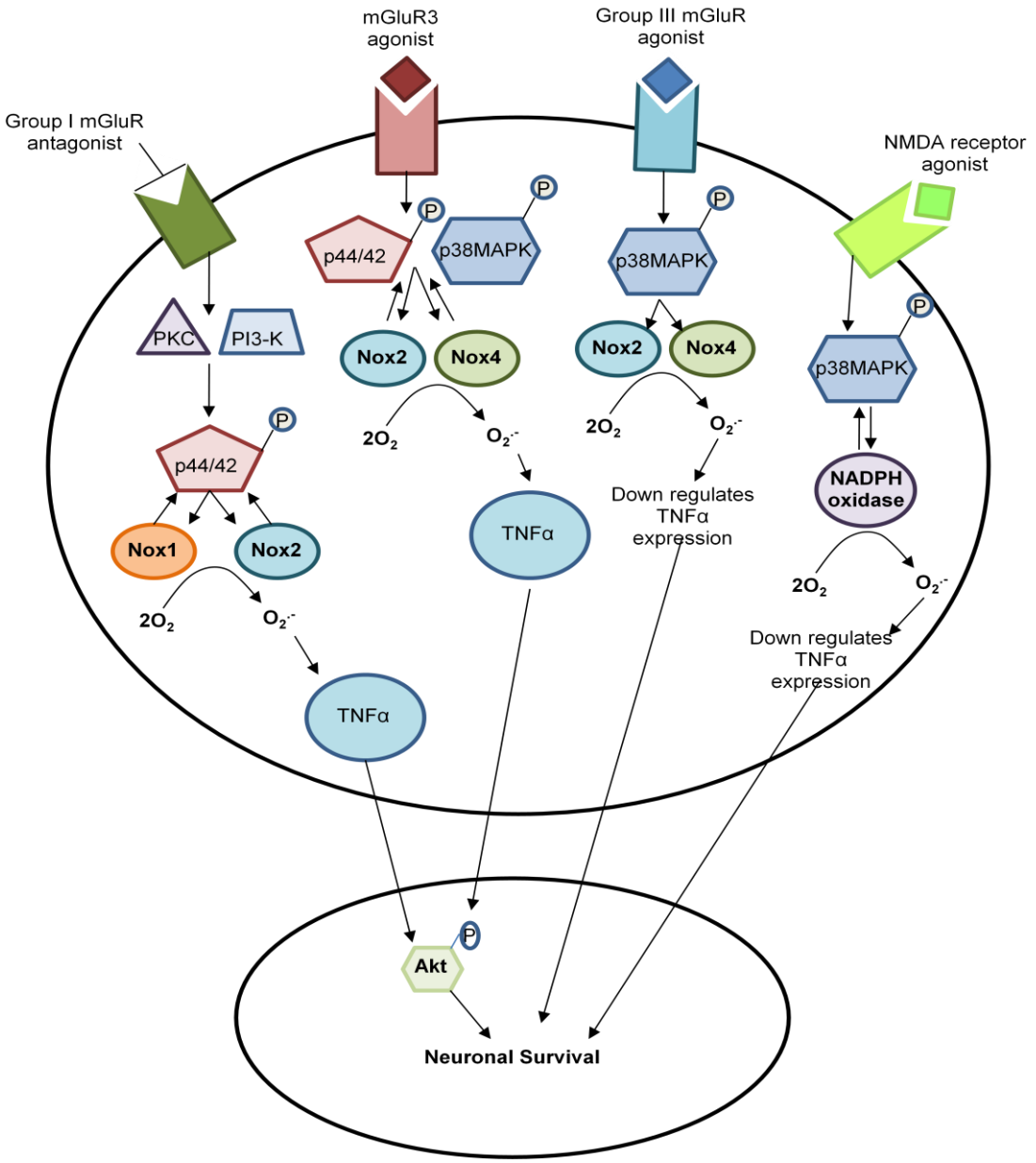

Figure 6.1 Microglial glutamate receptor induced superoxide productions and ramifications for neuronal survival. *The data presented here show that treatment of primary microglia with the mGluR group I receptor antagonist induces PKC and PI3-K activation, which could induce p44/42ERK activation and Nox1 and Nox2 mediated superoxide production. Furthermore, MTEP induced superoxide production also mediated the phosphorylation of p44/42ERK. Subsequent superoxide production induced the expression of TNFα, which correlated with an increase in neuronal Akt phosphorylation and subsequent neuronal survival. Activation of the microglial mGluR3 induced the production of superoxide through Nox2 and Nox4 through activation of the p38MAPK and p44/42ERK, which could also mediate phosphorylation of these signalling molecules. Nox2 and Nox4 activation also mediated TNFα expression which was shown to induce neuronal Akt phosphprylation and neuronal survival. Treatment of microglia with the mGluR group III agonist induced p38MAPK activation and Nox2 and Nox4 activation, which down-regulated TNFα expression and mediated neuronal survival. Finally, activation of the microglial NMDA receptor was shown induced the activation of the NADPH oxidase in a p38MAPK dependent manner, and superoxide production could also regulate p38MAPK phosphorylation. This also resulted in neuronal survival.*

Treatment of microglia with the $GABA_A$ receptor agonist or the P2Y2/4 receptor agonist was neurotoxic through activation of the NADPH oxidase (Fig. 6.2, Table 8). Treatment of microglia with the $GABA_A$ receptor agonist muscimol significantly increased superoxide production in a Nox1 dependent manner, which was dependent on PKC and p38MAPK signalling (Fig. 6.2). Furthermore, Nox1 induced superoxide production significantly elevated TNFα release, which correlated with increased neuronal apoptosis (Fig. 6.2).

Although this is the first time that it has been shown that modulation of the microglial $GABA_A$ receptor mediates superoxide production with neurotoxic consequences, it has been found that $GABA_A$ receptor expression is up-regulated on gliomas (Synowitz et al. 2001), suggesting a role in pathology. Furthermore, activation of the astrocytic $GABA_A$ receptor mediates IL-6 release in a p38MAPK dependent manner (Roach et al. 2008). IL-6 is released from human microglia expressing active Nox4 (Li et al. 2009), and muscimol regulates Nox4 activation in endothelial cells (Tyagi et al. 2009), therefore lending support to the findings here that activation of the microglial $GABA_A$ receptor may induce superoxide production in an NADPH oxidase dependent manner. Muscimol treatment was shown to induce Nox1 activation, which correlated with a neurotoxic phenotype, in agreement with Chéret et al. (2008) who show that microglial Nox1 activation induces the release of cytotoxic factors. These data therefore demonstrate that in diseases such as AD, in which the GABA tone is enhanced (Marczynski 1998), the elevated GABA levels may induce activation of microglial $GABA_A$ receptors which could perpetuate neurodegeneration.

Activation of the microglial P2Y2/4 receptor significantly elevated Nox1 and Nox2 mediated superoxide production in a PI3-K and p38MAPK dependent manner (Fig. 6.2, Table 8). Furthermore, this Nox1/2 mediated superoxide production induced p44/42ERK phosphorylation which is implicated in enhanced microglial activation (Thellung et al. 2007), and can perpetuate cytokine release and neuronal apoptosis. P2Y2/4 receptor induced

Nox1/2 activation induced TNFα expression, which correlated with neuronal apoptosis in an NADPH oxidase dependent manner (Fig. 6.2).

Activation of microglial P2Y receptors mediates cytokine release (Ogata et al. 2003) and promotes a phagocytic phenotype (Inoue 2002). Furthermore, the phagocytic phenotype is elicited following the leakage of UDP and UTP in the hippocampus after excitotoxicity (Inoue et al. 2009). It could therefore be suggested that microglial P2Y2/4 receptor activation in pathological conditions may favour microglial reactivity and neuronal death in an NADPH oxidase dependent manner, which may be dependent on p44/42ERK activation (Luke & Hexum 2008), shown to induce a reactive microglial phenotype and promote TNFα release, which contributes to neurodegenerative conditions (Hide 2003), in agreement with the data presented here. These data show that the upstream mediator of p44/42ERK- induced TNFα release is Nox1, which promotes the release of cytotoxic factors from microglia (Chéret et al. 2008). Furthermore, it has been shown *in* vivo that activation of the microglial P2Y receptors facilitates the migration of immune cells to the sites of neuronal injury (Boucsein et al. 2003), and activation of the NADPH oxidase in macrophages can regulate the immune response by activating T-cells (Hildeman et al. 2003), which could suggest an immune regulatory role for superoxide production as a consequence of activation of microglial P2Y2/4 receptors *in vivo*. These findings therefore suggest that activation of the microglial $GABA_A$ or the P2Y2/4 receptors mediates neurotoxicity through Nox1 activation (Fig. 6.2).

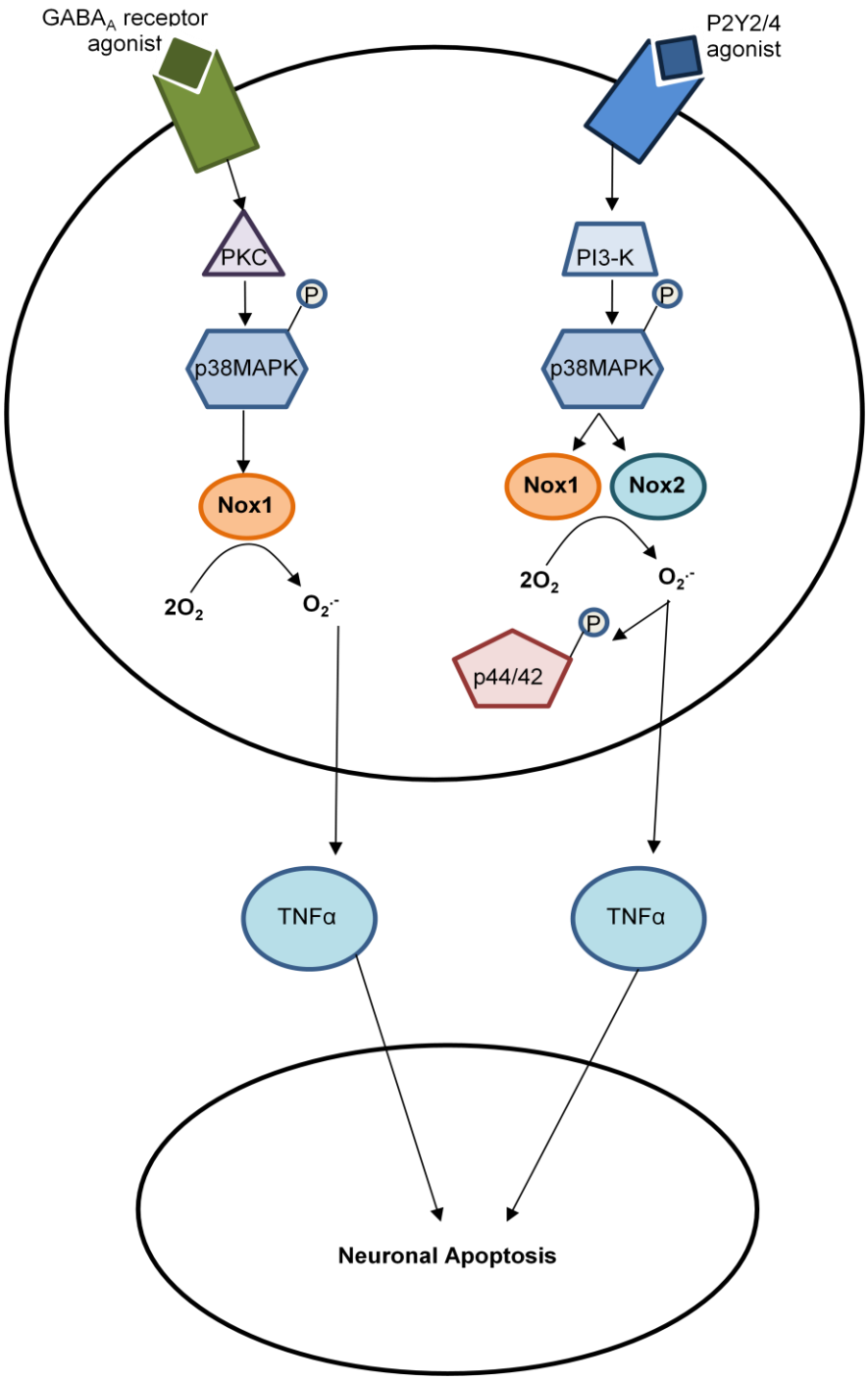

Figure 6.2 Activation of the microglial GABA$_A$ receptor or P2Y2/4 receptors are neurotoxic through activation of Nox1. *Treatment of microglia with the GABA$_A$ receptor agonist muscimol induced Nox1 activation through the activity of PKC and p38MAPK phosphorylation. This induced a significant increase in TNFα release which enhanced neuronal death in an NADPH oxidase dependent manner. Activation of the microglial P2Y2/4 receptor induced a significant increase in superoxide production through PI3-K and p38MAPK activation, which induced Nox1 and Nox2 activity, resulting in the release of TNFα and enhanced neuronal apoptosis, and also induced p44/42ERK phosphorylation in an NADPH oxidase dependent manner.*

Microglial neurotransmitter receptors modulated	Microglial NADPH oxidase isoform stimulated	Microglial Signalling cascades activated	Neuroprotective or neurotoxic consequences	Neuronal death / survival cascades activated
Group I mGluR antagonism	Nox1 and Nox2	PKC, PI3-K, p44/42ERK	Neuroprotective	Akt / Unknown
Group II mGluR3 activation	Nox2 and Nox4	p44/42ERK, p38MAPK	Neuroprotective	Akt / Unknown
Group III mGluR activation	Nox2 and Nox4	p38MAPK	Neuroprotective	Unknown
NMDA receptor activation	Unknown	p38MAPK	Neuroprotective	Unknown
$GABA_A$ receptor activation	Nox1	PKC, p38MAPK – mediates TNFα production and release	Neurotoxic	Caspase 12 cleavage
P2Y2/4 receptor activation	Nox1 and Nox2	PI3-K, p38MAPK – mediates TNFα production and release	Neurotoxic	Unknown

Table 8. Summary table of NADPH oxidase activation and signalling cascades elicited by modulation of microglial neurotransmitter receptors

6.2 Conclusions

The data presented here show that modulation of microglial neurotransmitter receptors mediates NADPH oxidase activation with consequences for neuronal survival. In line with published findings, the data in this thesis shows that treatment of microglia with the mGluR3 agonist NAAG (Taylor et al. 2002) and activation of the group III mGluR with L-AP4 (Taylor et al. 2003) is neuroprotective, which is mediated by Nox2 and Nox4. Stimulation of the microglial Nox2 and Nox4 isoforms could therefore modulate the inflammatory response in the CNS, with protective consequences for neurodegeneration (Hultqvist et al. 2004; Groeger et al. 2009). Antagonism of the microglial group I mGluR also promoted neuroprotection through Nox2. In addition, activation of the microglial NMDA receptor was neuroprotective in line with published findings showing that the NMDA receptor induces the release of neurotrophic factors from microglia (Liang et al. 2010). Furthermore, it could be

suggested that microglia take up excess glutamate released in degenerative conditions through the mGluR and NMDA receptors to facilitate neuronal survival. This could represent a mechanism in which microglial Nox2 and Nox4 activation mediate neuroprotection following exposure to glutamate in conditions such as AD and ischaemia, in which high levels of glutamate are found in the extracellular space. Modulation of these glutamate receptors could therefore represent a protective mechanism through activation of Nox2, which is protective in other systemic inflammatory conditions (Hultqvist et al. 2009).

In contrast, microglial $GABA_A$ and P2Y2/4 receptor activation was neurotoxic through Nox1 activation and TNFα release in an NADPH oxidase dependent manner. Reports have shown that the $GABA_A$ receptor is up-regulated on gliomas (Synowitz et al. 2001), representative of microglial reactivity, and high levels of GABA are present in the CNS following stroke (Hutchinson et al. 2002), and in the AD brain (Marczynski 1998) suggesting that neuronal damage may be facilitated through GABA activation of microglia and subsequent Nox1 activation. Furthermore, GABA perpetuates the cytotoxicity of immunocompetent cells (Bergeret et al. 1998), and the findings here suggest that this may be dependent on NADPH oxidase activation. Activation of the microglial P2Y receptors mediates TNFα expression and promotes a phagocytic phenotype (Koizumi et al. 2007). Furthermore, as UTP is released from hippocampal neurons following excitotoxicity (Inoue 2009), it could be suggested that the subsequent neurotoxicity is a consequence of P2Y2/4 mediated Nox1 activation, and inhibition of the microglial P2Y2/4 receptor may therefore be protective against degenerative conditions. Antagonism of the microglial $GABA_A$ receptor could also be protective through inhibition of Nox1 activation in conditions such as AD and ischaemia.

This thesis therefore shows that modulation of microglial glutamate receptors represents a neuroprotective therapy for the treatment of neurodegenerative conditions through activation of Nox2, whilst activation of the microglial $GABA_A$ and P2Y2/4 receptors could perpetuate

neurodegeneration through activation of the neurotoxic Nox1 isoform of the NADPH oxidase.

6.3 Suggested future work

Whilst the work presented here details the mechanism behind superoxide production and NADPH oxidase isoform activation as a consequence of modulation of microglial neurotransmitter receptors, further investigations could be performed into the mechanisms behind neuronal survival or death. A more comprehensive investigation into the factors released from microglia following treatment with neurotransmitter receptor modulators in an NADPH oxidase manner should be performed using a proteomic approach, to provide further information on which cytokines or released factors are responsible for modulating neuronal survival. Furthermore, a detailed investigation into the effects of microglial neurotransmitter receptor modulation, and subsequent NADPH oxidase activation on neuronal survival in a more physiological setting should be conducted; for example, investigating the effects of microglial NADPH oxidase modulation on neuronal survival after exposure to Aβ, CgA or myelin. Following on from these studies, an *in vivo* system should be used to analyse the effects of modulation of the microglial neurotransmitter receptors and subsequent NADPH oxidase activation in animal models of neurodegenerative disease. In addition, it would be interesting to see whether microglial neurotransmitter receptor induced NADPH oxidase activation regulates the activation of other immune cells, or a disruption of the BBB, to mediate the activity of the peripheral immune system, which could have important ramifications for attenuating disease progression. It would be important to see whether the data presented here translates to human cells, whereby human macrophages could be used. These studies would be beneficial to build upon the work presented here and to investigate the hypothesis that modulation of microglial neurotransmitter receptors could mediate

neuronal survival in an NADPH oxidase dependent manner in greater detail and in a more physiologically relevant setting.

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List of conferences attended and posters presented

- The 9th European meeting on glial cells in health and disease – Paris, September 2009. "Neurotransmitter modulation of superoxide production in microglia".
- The Society for Neuroscience – Chicago, October 2009. "Neurotransmitter modulation of superoxide production in microglia".
- The Queen Square Symposium – ION, March 2010. "Neurotransmitter receptor modulation of superoxide production in microglia".
- The UCL Neuroscience Symposium – UCL, April 2010. "Neurotransmitter receptor modulation of superoxide production in microglia".
- The International Symposium on the Pathophysiology of Reactive Oxygen and Nitrogen Species – Salamanca, May 2010. "Neurotransmitter receptor modulation of superoxide production in microglia".
- The UCL Neuroscience Symposium – UCL, July 2011. "Ionotropic glutamate receptors mediate superoxide production in microglia."